POLYKETIDE SYNTHASE ENZYMES AND RECOMBINANT DNA CONSTRUCTS THEREFOR

Cross-Reference to Related Applications

The present application claims priority to related U.S. patent application Serial Nos. 60/102,748, filed 2 Oct. 1998; 60/139,650, filed 17 June 1999; and 60/123,810, filed 11 Mar. 1999, each of which is incorporated herein by reference.

Field of the Invention

The present invention relates to polyketides and the polyketide synthase (PKS) enzymes that produce them. The invention also relates generally to genes encoding PKS enzymes and to recombinant host cells containing such genes and in which expression of such genes leads to the production of polyketides. The present invention also relates to compounds useful as medicaments having immunosuppressive and/or neurotrophic activity. Thus, the invention relates to the fields of chemistry, molecular biology, and agricultural, medical, and veterinary technology.

Background of the Invention

Polyketides are a class of compounds synthesized from 2-carbon units through a series of condensations and subsequent modifications. Polyketides occur in many types of organisms, including fungi and mycelial bacteria, in particular, the actinomycetes. Polyketides are biologically active molecules with a wide variety of structures, and the class encompasses numerous compounds with diverse activities. Tetracycline, erythromycin, epothilone, FK-506, FK-520, narbomycin, picromycin, rapamycin, spinocyn, and tylosin are examples of polyketides. Given the difficulty in producing polyketide compounds by traditional chemical methodology, and the typically low production of polyketides in wild-type cells, there has been considerable interest in finding improved or alternate means to produce polyketide compounds.

This interest has resulted in the cloning, analysis, and manipulation by recombinant DNA technology of genes that encode PKS enzymes. The resulting technology allows one to manipulate a known PKS gene cluster either to produce the polyketide synthesized by that PKS at higher levels than occur in nature or in hosts that otherwise do not produce the polyketide. The technology also allows one to produce molecules that are structurally related to, but distinct from, the polyketides produced from known PKS gene clusters. See, e.g., PCT publication Nos. WO 93/13663; 95/08548; 96/40968; 97/02358; 98/27203; and 98/49315; United States Patent Nos. 4,874,748; 5,063,155; 5,098,837; 5,149,639; 5,672,491; 5,712,146; 5,830,750; and 5,843,718; and Fu *et al.*, 1994, *Biochemistry 33*: 9321-9326; McDaniel *et al.*, 1993, *Science 262*: 1546-1550; and Rohr, 1995, *Angew. Chem. Int. Ed. Engl. 34*(8): 881-888, each of which is incorporated herein by reference.

Polyketides are synthesized in nature by.PKS enzymes. These enzymes, which are complexes of multiple large proteins, are similar to the synthases that catalyze condensation of 2-carbon units in the biosynthesis of fatty acids. PKSs catalyze the biosynthesis of polyketides through repeated, decarboxylative Claisen condensations between acylthioester building blocks. The building blocks used to form complex polyketides are typically acylthioesters, such as acetyl, butyryl, propionyl, malonyl, hydroxymalonyl, methylmalonyl, and ethylmalonyl CoA. Other building blocks include amino acid like acylthioesters. PKS enzymes that incorporate such building blocks include an activity that functions as an amino acid ligase (an AMP ligase) or as a non-ribosomal peptide synthetase (NRPS). Two major types of PKS enzymes are known; these differ in their composition and mode of synthesis of the polyketide synthesized. These two major types of PKS enzymes are commonly referred to as Type I or “modular” and Type II “iterative” PKS enzymes.

In the Type I or modular PKS enzyme group, a set of separate catalytic active sites (each active site is termed a "domain", and a set thereof is termed a "module") exists for each cycle of carbon chain elongation and modification in the polyketide synthesis pathway. The typical modular PKS is composed of several large polypeptides, which can be segregated from amino to carboxy termini into a loading module, multiple extender

modules, and a releasing (or thioesterase) domain. The PKS enzyme known as 6-deoxyerythronolide B synthase (DEBS) is a Type I PKS. In DEBS, there is a loading module, six extender modules, and a thioesterase (TE) domain. The loading module, six extender modules, and TE of DEBS are present on three separate proteins (designated DEBS-1, DEBS-2, and DEBS-3, with two extender modules per protein). Each of the DEBS polypeptides is encoded by a separate open reading frame (ORF) or gene; these genes are known as *eryAI*, *eryAII*, and *eryAIII*. See Caffrey *et al.*, 1992, *FEBS Letters 304*: 205, and U.S. Patent No. 5,824,513, each of which is incorporated herein by reference.

Generally, the loading module is responsible for binding the first building block used to synthesize the polyketide and transferring it to the first extender module. The loading module of DEBS consists of an acyltransferase (AT) domain and an acyl carrier protein (ACP) domain. Another type of loading module utilizes an inactivated ketosynthase (KS) domain and AT and ACP domains. This inactivated KS is in some instances called KS^Q, where the superscript letter is the abbreviation for the amino acid, glutamine, that is present instead of the active site cysteine required for ketosynthase activity. In other PKS enzymes, including the FK-506 PKS, the loading module incorporates an unusual starter unit and is composed of a CoA ligase like activity domain. In any event, the loading module recognizes a particular acyl-CoA (usually acetyl or propionyl but sometimes butyryl or other acyl-CoA) and transfers it as a thiol ester to the ACP of the loading module.

The AT on each of the extender modules recognizes a particular extender-CoA (malonyl or alpha-substituted malonyl, i.e., methylmalonyl, ethylmalonyl, and 2-hydroxymalonyl) and transfers it to the ACP of that extender module to form a thioester. Each extender module is responsible for accepting a compound from a prior module, binding a building block, attaching the building block to the compound from the prior module, optionally performing one or more additional functions, and transferring the resulting compound to the next module.

Each extender module of a modular PKS contains a KS, AT, ACP, and zero, one, two, or three domains that modify the beta-carbon of the growing polyketide chain. A

typical (non-loading) minimal Type I PKS extender module is exemplified by extender module three of DEBS, which contains a KS domain, an AT domain, and an ACP domain. These three domains are sufficient to activate a 2-carbon extender unit and attach it to the growing polyketide molecule. The next extender module, in turn, is responsible for attaching the next building block and transferring the growing compound to the next extender module until synthesis is complete.

Once the PKS is primed with acyl- and malonyl-ACPs, the acyl group of the loading module is transferred to form a thiol ester (trans-esterification) at the KS of the first extender module; at this stage, extender module one possesses an acyl-KS and a malonyl (or substituted malonyl) ACP. The acyl group derived from the loading module is then covalently attached to the alpha-carbon of the malonyl group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading building block (elongation or extension).

The polyketide chain, growing by two carbons each extender module, is sequentially passed as covalently bound thiol esters from extender module to extender module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the name polyketide arises. Most commonly, however, additional enzymatic activities modify the beta keto group of each two carbon unit just after it has been added to the growing polyketide chain but before it is transferred to the next module.

Thus, in addition to the minimal module containing KS, AT, and ACP domains necessary to form the carbon-carbon bond, and as noted above, other domains that modify the beta-carbonyl moiety can be present. Thus, modules may contain a ketoreductase (KR) domain that reduces the keto group to an alcohol. Modules may also contain a KR domain plus a dehydratase (DH) domain that dehydrates the alcohol to a double bond. Modules may also contain a KR domain, a DH domain, and an enoylreductase (ER) domain that converts the double bond product to a saturated single bond using the beta carbon as a methylene function. An extender module can also contain other enzymatic activities, such as, for example, a methylase or dimethylase activity.

After traversing the final extender module, the polyketide encounters a releasing domain that cleaves the polyketide from the PKS and typically cyclizes the polyketide. For example, final synthesis of 6-dEB is regulated by a TE domain located at the end of extender module six. In the synthesis of 6-dEB, the TE domain catalyzes cyclization of the macrolide ring by formation of an ester linkage. In FK-506, FK-520, rapamycin, and similar polyketides, the TE activity is replaced by a RapP (for rapamycin) or RapP like activity that makes a linkage incorporating a pipecolate acid residue. The enzymatic activity that catalyzes this incorporation for the rapamycin enzyme is known as RapP, encoded by the *rapP* gene. The polyketide can be modified further by tailoring enzymes; these enzymes add carbohydrate groups or methyl groups, or make other modifications, i.e., oxidation or reduction, on the polyketide core molecule. For example, 6-dEB is hydroxylated at C-6 and C-12 and glycosylated at C-3 and C-5 in the synthesis of erythromycin A.

In Type I PKS polypeptides, the order of catalytic domains is conserved. When all beta-keto processing domains are present in a module, the order of domains in that module from N-to-C-terminus is always KS, AT, DH, ER, KR, and ACP. Some or all of the beta-keto processing domains may be missing in particular modules, but the order of the domains present in a module remains the same. The order of domains within modules is believed to be important for proper folding of the PKS polypetides into an active complex. Importantly, there is considerable flexibility in PKS enzymes, which allows for the genetic engineering of novel catalytic complexes. The engineering of these enzymes is achieved by modifying, adding, or deleting domains, or replacing them with those taken from other Type I PKS enzymes. It is also achieved by deleting, replacing, or adding entire modules with those taken from other sources. A genetically engineered PKS complex should of course have the ability to catalyze the synthesis of the product predicted from the genetic alterations made.

Alignments of the many available amino acid sequences for Type I PKS enzymes has approximately defined the boundaries of the various catalytic domains. Sequence alignments also have revealed linker regions between the catalytic domains and at the N- and C-termini of individual polypeptides. The sequences of these linker regions are less

well conserved than are those for the catalytic domains, which is in part how linker regions are identified. Linker regions can be important for proper association between domains and between the individual polypeptides that comprise the PKS complex. One can thus view the linkers and domains together as creating a scaffold on which the domains and modules are positioned in the correct orientation to be active. This organization and positioning, if retained, permits PKS domains of different or identical substrate specificities to be substituted (usually at the DNA level) between PKS enzymes by various available methodologies. In selecting the boundaries of, for example, an AT replacement, one can thus make the replacement so as to retain the linkers of the recipient PKS or to replace them with the linkers of the donor PKS AT domain, or, preferably, make both constructs to ensure that the correct linker regions between the KS and AT domains have been included in at least one of the engineered enzymes. Thus, there is considerable flexibility in the design of new PKS enzymes with the result that known polyketides can be produced more effectively, and novel polyketides useful as pharmaceuticals or for other purposes can be made.

By appropriate application of recombinant DNA technology, a wide variety of polyketides can be prepared in a variety of different host cells provided one has access to nucleic acid compounds that encode PKS proteins and polyketide modification enzymes. The present invention helps meet the need for such nucleic acid compounds by providing recombinant vectors that encode the FK-520 PKS enzyme and various FK-520 modification enzymes. Moreover, while the FK-506 and FK-520 polyketides have many useful activities, there remains a need for compounds with similar useful activities but with better pharmacokinetic profile and metabolism and fewer side-effects. The present invention helps meet the need for such compounds as well.

Summary of the Invention

In one embodiment, the present invention provides recombinant DNA vectors that encode all or part of the FK-520 PKS enzyme. Illustrative vectors of the invention include cosmid pKOS034-120, pKOS034-124, pKOS065-C31, pKOS065-C3, pKOS065-M27, and pKOS065-M21. The invention also provides nucleic acid compounds that

encode the various domains of the FK-520 PKS, i.e., the KS, AT, ACP, KR, DH, and ER domains. These compounds can be readily used, alone or in combination with nucleic acids encoding other FK-520 or non-FK-520 PKS domains, as intermediates in the construction of recombinant vectors that encode all or part of PKS enzymes that make novel polyketides.

The invention also provides isolated nucleic acids that encode all or part of one or more modules of the FK-520 PKS, each module comprising a ketosynthase activity, an acyl transferase activity, and an acyl carrier protein activity. The invention provides an isolated nucleic acid that encodes one or more open reading frames of FK-520 PKS genes, said open reading frames comprising coding sequences for a CoA ligase activity, an NRPS activity, or two or more extender modules. The invention also provides recombinant expression vectors containing these nucleic acids.

In another embodiment, the invention provides isolated nucleic acids that encode all or a part of a PKS that contains at least one module in which at least one of the domains in the module is a domain from a non-FK-520 PKS and at least one domain is from the FK-520 PKS. The non-FK-520 PKS domain or module originates from the rapamycin PKS, the FK-506 PKS, DEBS, or another PKS. The invention also provides recombinant expression vectors containing these nucleic acids.

In another embodiment, the invention provides a method of preparing a polyketide, said method comprising transforming a host cell with a recombinant DNA vector that encodes at least one module of a PKS, said module comprising at least one FK-520 PKS domain, and culturing said host cell under conditions such that said PKS is produced and catalyzes synthesis of said polyketide. In one aspect, the method is practiced with a *Streptomyces* host cell. In another aspect, the polyketide produced is FK-520. In another aspect, the polyketide produced is a polyketide related in structure to FK-520. In another aspect, the polyketide produced is a polyketide related in structure to FK-506 or rapamycin.

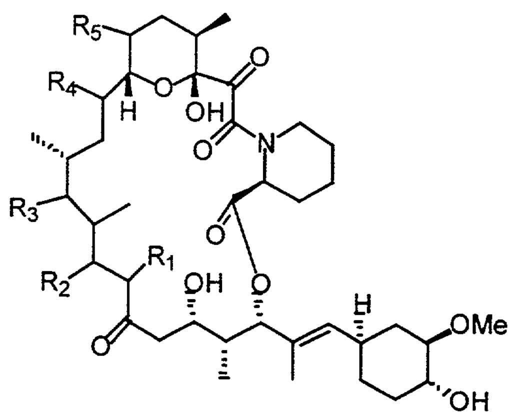
In another embodiment, the invention provides a set of genes in recombinant form sufficient for the synthesis of ethylmalonyl CoA in a heterologous host cell. These genes and the methods of the invention enable one to create recombinant host cells with the

ability to produce polyketides or other compounds that require ethylmalonyl CoA for biosynthesis. The invention also provides recombinant nucleic acids that encode AT domains specific for ethylmalonyl CoA. Thus, the compounds of the invention can be used to produce polyketides requiring ethylmalonyl CoA in host cells that otherwise are unable to produce such polyketides.

In another embodiment, the invention provides a set of genes in recombinant form sufficient for the synthesis of 2-hydroxymalonyl CoA and 2-methoxymalonyl CoA in a heterologous host cell. These genes and the methods of the invention enable one to create recombinant host cells with the ability to produce polyketides or other compounds that require 2-hydroxymalonyl CoA for biosynthesis. The invention also provides recombinant nucleic acids that encode AT domains specific for 2-hydroxymalonyl CoA and 2-methoxymalonyl CoA. Thus, the compounds of the invention can be used to produce polyketides requiring 2-hydroxymalonyl CoA or 2-methoxymalonyl CoA in host cells that are otherwise unable to produce such polyketides.

In another embodiment, the invention provides a compound related in structure to FK-520 or FK-506 that is useful in the treatment of a medical condition. These compounds include compounds in which the C-13 methoxy group is replaced by a moiety selected from the group consisting of hydrogen, methyl, and ethyl moieties. Such compounds are less susceptible to the main *in vivo* pathway of degradation for FK-520 and FK-506 and related compounds and thus exhibit an improved pharmacokinetic profile. The compounds of the invention also include compounds in which the C-15 methoxy group is replaced by a moiety selected from the group consisting of hydrogen, methyl, and ethyl moieties. The compounds of the invention also include the above compounds further modified by chemical methodology to produce derivatives such as, but not limited to, the C-18 hydroxyl derivatives, which have potent neurotrophin but not immunosuppresion activities.

Thus, the invention provides polyketides having the structure:



wherein, R_1 is hydrogen, methyl, ethyl, or allyl; R_2 is hydrogen or hydroxyl, provided that when R_2 is hydrogen, there is a double bond between C-20 and C-19; R_3 is hydrogen or hydroxyl; R_4 is methoxyl, hydrogen, methyl, or ethyl; and R_5 is methoxyl, hydrogen, methyl, or ethyl; but not including FK-506, FK-520, 18-hydroxy-FK-520, and 18-hydroxy-FK-506. The invention provides these compounds in purified form and in pharmaceutical compositions.

In another embodiment, the invention provides a method for treating a medical condition by administering a pharmaceutically efficacious dose of a compound of the invention. The compounds of the invention may be administered to achieve immunosuppression or to stimulate nerve growth and regeneration.

These and other embodiments and aspects of the invention will be more fully understood after consideration of the attached Drawings and their brief description below, together with the detailed description, examples, and claims that follow.

Brief Description of the Drawings

Figure 1 shows a diagram of the FK-520 biosynthetic gene cluster. The top line provides a scale in kilobase pairs (kb). The second line shows a restriction map with selected restriction enzyme recognition sequences indicated. K is *Kpn*I; X is *Xho*I, S is *Sac*I; P is *Pst*I; and E is *Eco*RI. The third line indicates the position of FK-520 PKS and related genes. Genes are abbreviated with a one letter designation, i.e., C is *fkbC*.

Immediately under the third line are numbered segments showing where the loading module (L) and ten different extender modules (numbered 1 - 10) are encoded on the various genes shown. At the bottom of the Figure, the DNA inserts of various cosmids of the invention (i.e., 34-124 is cosmid pKOS034-124) are shown in alignment with the FK-520 biosynthetic gene cluster.

Figure 2 shows the loading module (load), the ten extender modules, and the peptide synthetase domain of the FK-520 PKS, together with, on the top line, the genes that encode the various domains and modules. Also shown are the various intermediates in FK-520 biosynthesis, as well as the structure of FK-520, with carbons 13, 15, 21, and 31 numbered. The various domains of each module and subdomains of the loading module are also shown. The darkened circles showing the DH domains in modules 2, 3, and 4 indicate that the dehydratase domain is not functional as a dehydratase; this domain may affect the stereochemistry at the corresponding position in the polyketide. The substituents on the FK-520 structure that result from the action of non-PKS enzymes are also indicated by arrows, together with the types of enzymes or the genes that code for the enzymes that mediate the action. Although the methyltransferase is shown acting at the C-13 and C-15 hydroxyl groups after release of the polyketide from the PKS, the methyltransferase may act on the 2-hydroxymalonyl substrate prior to or contemporaneously with its incorporation during polyketide synthesis.

Figure 3 shows a close-up view of the left end of the FK-520 gene cluster, which contains at least ten additional genes. The ethyl side chain on carbon 21 of FK-520 (Figure 2) is derived from an ethylmalonyl CoA extender unit that is incorporated by an ethylmalonyl specific AT domain in extender module 4 of the PKS. At least four of the genes in this region code for enzymes involved in ethylmalonyl biosynthesis. The polyhydroxybutyrate depolymerase is involved in maintaining hydroxybutyryl-CoA pools during FK-520 production. Polyhydroxybutyrate accumulates during vegetative growth and disappears during stationary phase in other *Streptomyces* (Ranade and Vining, 1993, *Can. J. Microbiol. 39*:377). Open reading frames with unknown function are indicated with a question mark.

Figure 4 shows a biosynthetic pathway for the biosynthesis of ethylmalonyl CoA from acetoacetyl CoA consistent with the function assigned to four of the genes in the FK-520 gene cluster shown in Figure 3.

Figure 5 shows a close-up view of the right-end of the FK-520 PKS gene cluster (and of the sequences on cosmid pKOS065-C31). The genes shown include *fkbD*, *fkbM* (a methyl transferase that methylates the hydroxyl group on C-31 of FK-520), *fkbN* (a homolog of a gene described as a regulator of cholesterol oxidase and that is believed to be a transcriptional activator), *fkbQ* (a type II thioesterase, which can increase polyketide production levels), and *fkbS* (a crotonyl-CoA reductase involved in the biosynthesis of ethylmalonyl CoA).

Figure 6 shows the proposed degradative pathway for tacrolimus (FK-506) metabolism.

Figure 7 shows a schematic process for the construction of recombinant PKS genes of the invention that encode PKS enzymes that produce 13-desmethoxy FK-506 and FK-520 polyketides of the invention, as described in Example 4, below.

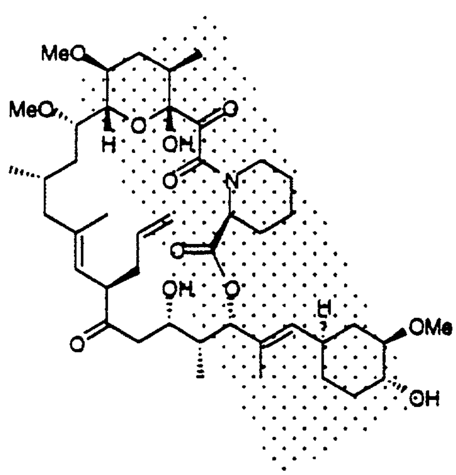
Figure 8, in Parts A and B, shows certain compounds of the invention preferred for dermal application in Part A and a synthetic route for making those compounds in Part B.

Detailed Description of the Invention

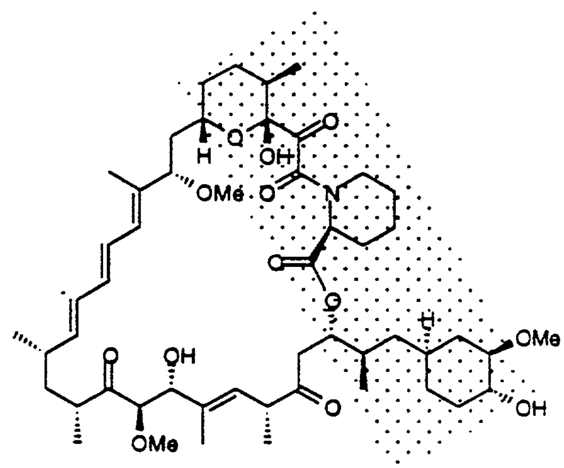
Given the valuable pharmaceutical properties of polyketides, there is a need for methods and reagents for producing large quantities of polyketides, as well as for producing related compounds not found in nature. The present invention provides such methods and reagents, with particular application to methods and reagents for producing the polyketides known as FK-520, also known as ascomycin or L-683,590 (see Holt *et al.*, 1993, *JACS 115*:9925), and FK-506, also known as tacrolimus. Tacrolimus is a macrolide immunosuppressant used to prevent or treat rejection of transplanted heart, kidney, liver, lung, pancreas, and small bowel allografts. The drug is also useful for the prevention and treatment of graft-versus-host disease in patients receiving bone marrow transplants, and for the treatment of severe, refractory uveitis. There have been additional

reports of the unapproved use of tacrolimus for other conditions, including alopecia universalis, autoimmune chronic active hepatitis, inflammatory bowel disease, multiple sclerosis, primary biliary cirrhosis, and scleroderma. The invention provides methods and reagents for making novel polyketides related in structure to FK-520 and FK-506, and structurally related polyketides such as rapamycin.

The FK-506 and rapamycin polyketides are potent immunosuppressants, with chemical structures shown below.



FK-506

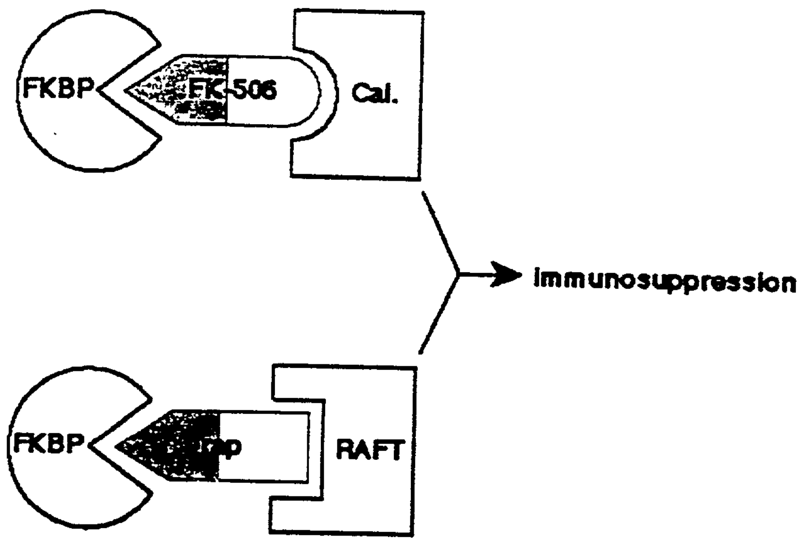


Rapamycin

FK-520 differs from FK-506 in that it lacks the allyl group at C-21 of FK-506, having instead an ethyl group at that position, and has similar activity to FK-506, albeit reduced immunosuppressive activity.

These compounds act through initial formation of an intermediate complex with protein "immunophilins" known as FKBPs (FK-506 binding proteins), including FKBP-12. Immunophilins are a class of cytosolic proteins that form complexes with molecules such as FK-506, FK-520, and rapamycin that in turn serve as ligands for other cellular targets involved in signal transduction. Binding of FK-506, FK-520, and rapamycin to FKBP occurs through the structurally similar segments of the polyketide molecules, known as the "FKBP-binding domain" (as generally but not precisely indicated by the stippled regions in the structures above). The FK-506-FKBP complex then binds calcineurin, while the rapamycin-FKBP complex binds to a protein known as RAFT-1.

Binding of the FKBP-polyketide complex to these second proteins occurs through the dissimilar regions of the drugs known as the "effector" domains.



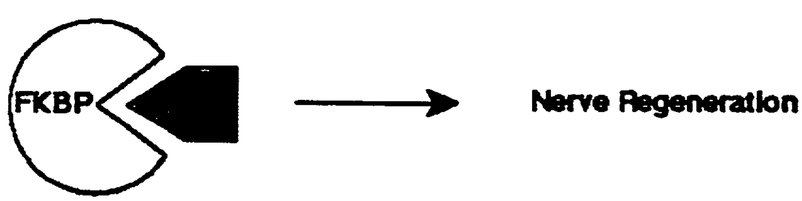
The three component FKBP-polyketide-effector complex is required for signal transduction and subsequent immunosuppressive activity of FK-506, FK-520, and rapamycin. Modifications in the effector domains of FK-506, FK-520, and rapamycin that destroy binding to the effector proteins (calcineurin or RAFT) lead to loss of immunosuppressive activity, even though FKBP binding is unaffected. Further, such analogs antagonize the immunosuppressive effects of the parent polyketides, because they compete for FKBP. Such non-immunosuppressive analogs also show reduced toxicity (see Dumont *et al.*, 1992, *Journal of Experimental Medicine 176*, 751-760), indicating that much of the toxicity of these drugs is not linked to FKBP binding.

In addition to immunosuppressive activity, FK-520, FK-506, and rapamycin have neurotrophic activity. In the central nervous system and in peripheral nerves, immunophilins are referred to as "neuroimmunophilins". The neuroimmunophilin FKBP is markedly enriched in the central nervous system and in peripheral nerves. Molecules that bind to the neuroimmunophilin FKBP, such as FK-506 and FK-520, have the remarkable effect of stimulating nerve growth. *In vitro*, they act as neurotrophins, i.e.,

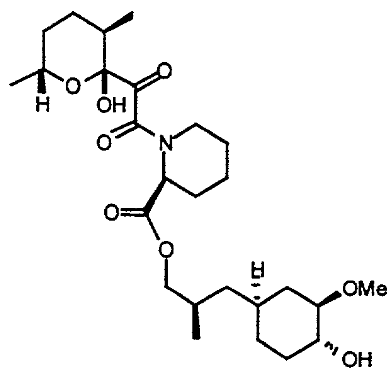
they promote neurite outgrowth in NGF-treated PC12 cells and in sensory neuronal cultures, and in intact animals, they promote regrowth of damaged facial and sciatic nerves, and repair lesioned serotonin and dopamine neurons in the brain. See Gold *et al.*, Jun. 1999, *J. Pharm. Exp. Ther. 289*(3): 1202-1210; Lyons *et al.*, 1994, *Proc. National Academy of Science 91*: 3191-3195; Gold *et al.*, 1995, *Journal of Neuroscience 15*: 7509-7516; and Steiner *et al.*, 1997, *Proc. National Academy of Science 94*: 2019-2024. Further, the restored central and peripheral neurons appear to be functional.

Compared to protein neurotrophic molecules (BNDF, NGF, etc.), the small-molecule neurotrophins such as FK-506, FK-520, and rapamycin have different, and often advantageous, properties. First, whereas protein neurotrophins are difficult to deliver to their intended site of action and may require intra-cranial injection, the small-molecule neurotrophins display excellent bioavailability; they are active when administered subcutaneously and orally. Second, whereas protein neurotrophins show quite specific effects, the small-molecule neurotrophins show rather broad effects. Finally, whereas protein neurotrophins often show effects on normal sensory nerves, the small-molecule neurotrophins do not induce aberrant sprouting of normal neuronal processes and seem to affect damaged nerves specifically. Neuroimmunophilin ligands have potential therapeutic utility in a variety of disorders involving nerve degeneration (e.g. multiple sclerosis, Parkinson's disease, Alzheimer's disease, stroke, traumatic spinal cord and brain injury, peripheral neuropathies).

Recent studies have shown that the immunosuppressive and neurite outgrowth activity of FK-506, FK-520, and rapamycin can be separated; the neuroregenerative activity in the absence of immunosuppressive activity is retained by agents which bind to FKBP but not to the effector proteins calcineurin or RAFT. See Steiner *et al.*, 1997, *Nature Medicine 3*: 421-428.



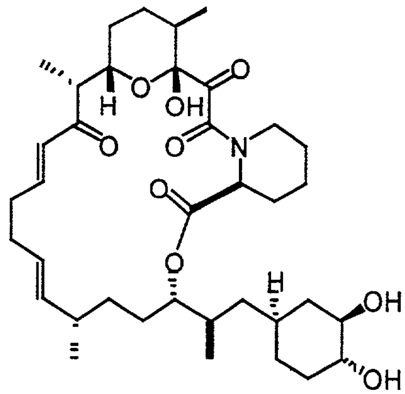
Available structure-activity data show that the important features for neurotrophic activity of rapamycin, FK-520, and FK-506 lie within the common, contiguous segments of the macrolide ring that bind to FKBP. This portion of the molecule is termed the "FKBP binding domain" (see VanDuyne *et al.*, 1993, *Journal of Molecular Biology 229*: 105-124.). Nevertheless, the effector domains of the parent macrolides contribute to conformational rigidity of the binding domain and thus indirectly contribute to FKBP binding.



"FKBP binding domain"

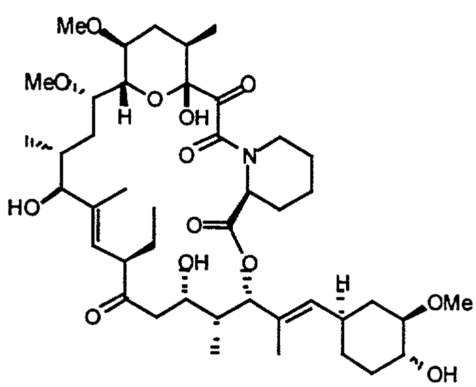
There are a number of other reported analogs of FK-506, FK-520, and rapamycin that bind to FKBP but not the effector protein calcineurin or RAFT. These analogs show effects on nerve regeneration without immunosuppressive effects.

Naturally occurring FK-520 and FK-506 analogs include the antascomycins, which are FK-506-like macrolides that lack the functional groups of FK-506 that bind to calcineurin (see Fehr *et al.*, 1996, *The Journal of Antibiotics 49*: 230-233). These molecules bind FKBP as effectively as does FK-506; they antagonize the effects of both FK-506 and rapamycin, yet lack immunosuppressive activity.

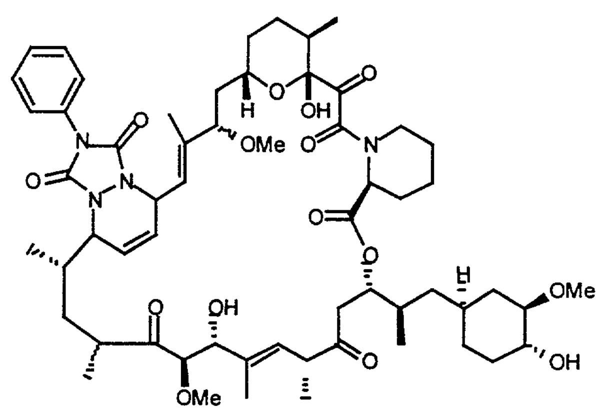


Antascomycin A

Other analogs can be produced by chemically modifying FK-506, FK-520, or rapamycin. One approach to obtaining neuroimmunophilin ligands is to destroy the effector binding region of FK-506, FK-520, or rapamycin by chemical modification. While the chemical modifications permitted on the parent compounds are quite limited, some useful chemically modified analogs exist. The FK-520 analog L-685,818 (ED_{50} = 0.7 nM for FKBP binding; see Dumont *et al.*, 1992), and the rapamycin analog WAY-124,466 (IC_{50} = 12.5 nM; see Ocain *et al.*, 1993, *Biochemistry Biophysical Research Communications 192*: 1340-134693) are about as effective as FK-506, FK-520, and rapamycin at promoting neurite outgrowth in sensory neurons (see Steiner *et al.*, 1997).



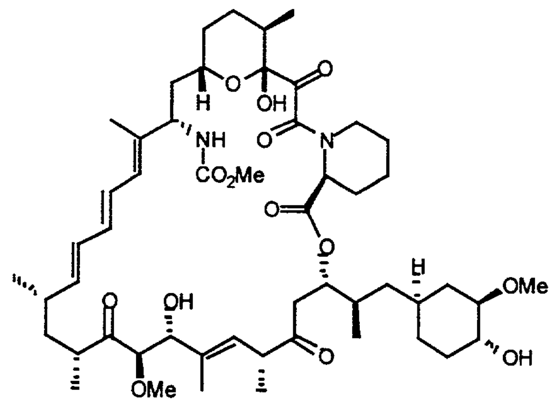
L-685,818



WAY-124,466

One of the few positions of rapamycin that is readily amenable to chemical modification is the allylic 16-methoxy group; this reactive group is readily exchanged by

acid-catalyzed nucleophilic substitution. Replacement of the 16-methoxy group of rapamycin with a variety of bulky groups has produced analogs showing selective loss of immunosuppressive activity while retaining FKBP-binding (see Luengo *et al.*, 1995, *Chemistry & Biology 2*: 471-481). One of the best compounds, **1**, below, shows complete loss of activity in the splenocyte proliferation assay with only a 10-fold reduction in binding to FKBP.



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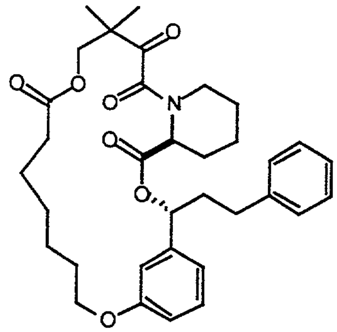
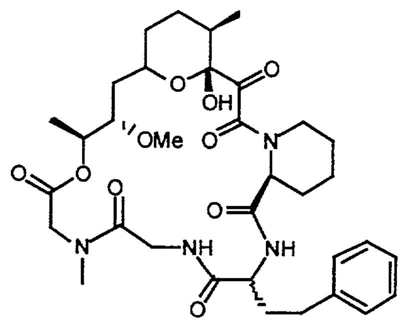
There are also synthetic analogs of FKBP binding domains. These compounds reflect an approach to obtaining neuroimmunophilin ligands based on "rationally designed" molecules that retain the FKBP-binding region in an appropriate conformation for binding to FKBP, but do not possess the effector binding regions. In one example, the ends of the FKBP binding domain were tethered by hydrocarbon chains (see Holt *et al.*, 1993, *Journal of the American Chemical Society 115*: 9925-9938); the best analog, **2**, below, binds to FKBP about as well as FK-506. In a similar approach, the ends of the FKBP binding domain were tethered by a tripeptide to give analog **3**, below, which binds to FKBP about 20-fold poorer than FK-506. These compounds are anticipated to have neuroimmunophilin binding activity.



2



3

In a primate MPTP model of Parkinson's disease, administration of FKBP ligand GPI-1046 caused brain cells to regenerate and behavioral measures to improve. MPTP is a neurotoxin, which, when administered to animals, selectively damages nigral-striatal dopamine neurons in the brain, mimicking the damage caused by Parkinson's disease. Whereas, before treatment, animals were unable to use affected limbs, the FKBP ligand restored the ability of animals to feed themselves and gave improvements in measures of locomotor activity, neurological outcome, and fine motor control. There were also corresponding increases in regrowth of damaged nerve terminals. These results demonstrate the utility of FKBP ligands for treatment of diseases of the CNS.

From the above description, two general approaches towards the design of non-immunosuppressant, neuroimmunophilin ligands can be seen. The first involves the construction of constrained cyclic analogs of FK-506 in which the FKBP binding domain is fixed in a conformation optimal for binding to FKBP. The advantages of this approach are that the conformation of the analogs can be accurately modeled and predicted by computational methods, and the analogs closely resemble parent molecules that have proven pharmacological properties. A disadvantage is that the difficult chemistry limits the numbers and types of compounds that can be prepared. The second approach involves the trial and error construction of acyclic analogs of the FKBP binding domain by conventional medicinal chemistry. The advantages to this approach are that the chemistry is suitable for production of the numerous compounds needed for such interactive chemistry-bioassay approaches. The disadvantages are that the molecular types of compounds that have emerged have no known history of appropriate pharmacological

properties, have rather labile ester functional groups, and are too conformationally mobile to allow accurate prediction of conformational properties.

The present invention provides useful methods and reagents related to the first approach, but with significant advantages. The invention provides recombinant PKS genes that produce a wide variety of polyketides that cannot otherwise be readily synthesized by chemical methodology alone. Moreover, the present invention provides polyketides that have either or both of the desired immunosuppressive and neurotrophic activities, some of which are produced only by fermentation and others of which are produced by fermentation and chemical modification. Thus, in one aspect, the invention provides compounds that optimally bind to FKBP but do not bind to the effector proteins. The methods and reagents of the invention can be used to prepare numerous constrained cyclic analogs of FK-520 in which the FKBP binding domain is fixed in a conformation optimal for binding to FKBP. Such compounds will show neuroimmunophilin binding (neurotrophic) but not immunosuppressive effects. The invention also allows direct manipulation of FK-520 and related chemical structures *via* genetic engineering of the enzymes involved in the biosynthesis of FK-520 (as well as related compounds, such as FK-506 and rapamycin); similar chemical modifications are simply not possible because of the complexity of the structures. The invention can also be used to introduce "chemical handles" into normally inert positions that permit subsequent chemical modifications.

Several general approaches to achieve the development of novel neuroimmunophilin ligands are facilitated by the methods and reagents of the present invention. One approach is to make "point mutations" of the functional groups of the parent FK-520 structure that bind to the effector molecules to eliminate their binding potential. These types of structural modifications are difficult to perform by chemical modification, but can be readily accomplished with the methods and reagents of the invention.

A second, more extensive approach facilitated by the present invention is to utilize molecular modeling to predict optimal structures *ab initio* that bind to FKBP but not effector molecules. Using the available X-ray crystal structure of FK-520 (or FK-506) bound to FKBP, molecular modeling can be used to predict polyketides that should

optimally bind to FKBP but not calcineurin. Various macrolide structures can be generated by linking the ends of the FKBP-binding domain with "all possible" polyketide chains of variable length and substitution patterns that can be prepared by genetic manipulation of the FK-520 or FK-506 PKS gene cluster in accordance with the methods of the invention. The ground state conformations of the virtual library can be determined, and compounds that possess binding domains most likely to bind well to FKBP can be prepared and tested.

Once a compound is identified in accordance with the above approaches, the invention can be used to generate a focused library of analogs around the lead candidate, to "fine tune" the compound for optimal properties. Finally, the genetic engineering methods of the invention can be directed towards producing "chemical handles" that enable medicinal chemists to modify positions of the molecule previously inert to chemical modification. This opens the path to previously prohibited chemical optimization of lead compounds by time-proven approaches.

Moreover, the present invention provides polyketide compounds and the recombinant genes for the PKS enzymes that produce the compounds that have significant advantages over FK-506 and FK-520 and their analogs. The metabolism and pharmacokinetics of tacrolimus has been exstensively studied, and FK-520 is believed to be similar in these respects. Absorption of tacrolimus is rapid, variable, and incomplete from the gastrointestinal tract (Harrison's Principles of Internal Medicine, 14th edition, 1998, McGraw Hill, 14, 20, 21, 64-67). The mean bioavailability of the oral dosage form is 27%, (range 5 to 65%). The volume of distribution (VolD) based on plasma is 5 to 65 L per kg of body weight (L/kg), and is much higher than the VolD based on whole blood concentrations, the difference reflecting the binding of tacrolimus to red blood cells. Whole blood concentrations may be 12 to 67 times the plasma concentrations. Protein binding is high (75 to 99%), primarily to albumin and alpha1-acid glycoprotein. The half-life for distribution is 0.9 hour; elimination is biphasic and variable: terminal-11.3 hr (range, 3.5 to 40.5 hours). The time to peak concentration is 0.5 to 4 hours after oral administration.

Tacrolimus is metabolized primarily by cytochrome P450 3A enzymes in the liver and small intestine. The drug is extensively metabolized with less than 1% excreted unchanged in urine. Because hepatic dysfunction decreases clearance of tacrolimus, doses have to be reduced substantially in primary graft non-function, especially in children. In addition, drugs that induce the cytochrome P450 3A enzymes reduce tacrolimus levels, while drugs that inhibit these P450s increase tacrolimus levels. Tacrolimus bioavailability doubles with co-administration of ketoconazole, a drug that inhibits P450 3A. See, Vincent *et al.*, 1992, *In vitro* metabolism of FK-506 in rat, rabbit, and human liver microsomes: Identification of a major metabolite and of cytochrome P450 3A as the major enzymes responsible for its metabolism, *Arch. Biochem. Biophys. 294*: 454-460; Iwasaki *et al.*, 1993, Isolation, identification, and biological activities of oxidative metabolites of FK-506, a potent immunosuppressive macrolide lactone, *Drug Metabolism & Disposition 21*: 971-977; Shiraga *et al.*, 1994, Metabolism of FK-506, a potent immunosuppressive agent, by cytochrome P450 3A enzymes in rat, dog, and human liver microsomes, *Biochem. Pharmacol. 47*: 727-735; and Iwasaki *et al.*, 1995, Further metabolism of FK-506 (Tacrolimus); Identification and biological activities of the metabolites oxidized at multiple sites of FK-506, *Drug Metabolism & Disposition 23*: 28-34. The cytochrome P450 3A subfamily of isozymes has been implicated as important in this degradative process.

Structures of the eight isolated metabolites formed by liver microsomes are shown in Figure 6. Four metabolites of FK-506 involve demethylation of the oxygens on carbons 13, 15, and 31, and hydroxylation of carbon 12. The 13-demethylated (hydroxy) compounds undergo cyclizations of the 13-hydroxy at C-10 to give MI, MVI and MVII, and the 12-hydroxy metabolite at C-10 to give I. Another four metabolites formed by oxidation of the four metabolites mentioned above were isolated by liver microsomes from dexamethasone treated rats. Three of these are metabolites doubly demethylated at the methoxy groups on carbons 15 and 31 (M-V), 13 and 31 (M-VI), and 13 and 15 (M-VII). The fourth, M-VIII, was the metabolite produced after demethylation of the 31-methoxy group, followed by formation of a fused ring system by further oxidation. Among the eight metabolites, M-II has immunosuppressive activity comparable to that of

FK-506, whereas the other metabolites exhibit weak or negligible activities. Importantly, the major metabolite of human, dog, and rat liver microsomes is the 13-demethylated and cyclized FK-506 (M-I).

Thus, the major metabolism of FK-506 proceeds via 13-demethylation followed by cyclization to the inactive M-I, this representing about 90% of the metabolic products after a 10 minute incubation with liver microsomes. Analogs of tacrolimus that do not possess a C-13 methoxy group would not be susceptible to the first and most important biotransformation in the destructive metabolism of tacrolimus (i.e. cyclization of 13-hydroxy to C-10). Thus, a 13-desmethoxy analog of FK-506 should have a longer half-life in the body than does FK-506. The C-13 methoxy group is believed not to be required for binding to FKBP or calcineurin. The C-13 methoxy is not present on the identical position of rapamycin, which binds to FKBP with equipotent affinity as tacrolimus. Also, analysis of the 3-dimensional structure of the FKBP-tacrolimus-calcineurin complex shows that the C-13 methoxy has no interaction with FKBP and only a minor interaction with calcineurin. The present invention provides C-13-desmethoxy analogs of FK-506 and FK-520, as well as the recombinant genes that encode the PKS enzymes that catalyze their synthesis and host cells that produce the compounds.

These compounds exhibit, relative to their naturally occurring counterparts, prolonged immunosuppressive action *in vivo*, thereby allowing a lower dosage and/or reduced frequency of administration. Dosing is more predictable, because the variability in FK-506 dosage is largely due to variation of metabolism rate. FK-506 levels in blood can vary widely depending on interactions with drugs that induce or inhibit cytochrome P450 3A (summarized in USP Drug Information for the Health Care Professional). Of particular importance are the numerous drugs that inhibit or compete for CYP 3A, because they increase FK-506 blood levels and lead to toxicity (Prograf package insert, Fujisawa□US, Rev 4/97, Rec 6/97). Also important are the drugs that induce P450 3A (e.g. Dexamethasone), because they decrease FK-506 blood levels and reduce efficacy. Because the major site of CYP 3A action on FK-506 is removed in the analogs provided by the present invention, those analogs are not as susceptible to drug interactions as the naturally occurring compounds.

Hyperglycemia, nephrotoxicity, and neurotoxicity are the most significant adverse effects resulting from the use of FK-506 and are believed to be similar for FK-520. Because these effects appear to occur primarily by the same mechanism as the immunosuppressive action (i.e. FKBP-calcineurin interaction), the intrinsic toxicity of the desmethoxy analogs may be similar to FK-506. However, toxicity of FK-506 is dose related and correlates with high blood levels of the drug (Prograf package insert, Fujisawa□US, Rev 4/97, Rec 6/97). Because the levels of the compounds provided by the present invention should be more controllable, the incidence of toxicity should be significantly decreased with the 13-desmethoxy analogs. Some reports show that certain FK-506 metabolites are more toxic than FK-506 itself, and this provides an additional reason to expect that a CYP 3A resistant analog can have lower toxicity and a higher therapeutic index.

Thus, the present invention provides novel compounds related in structure to FK-506 and FK-520 but with improved properties. The invention also provides methods for making these compounds by fermentation of recombinant host cells, as well as the recombinant host cells, the recombinant vectors in those host cells, and the recombinant proteins encoded by those vectors. The present invention also provides other valuable materials useful in the construction of these recombinant vectors that have many other important applications as well. In particular, the present invention provides the FK-520 PKS genes, as well as certain genes involved in the biosynthesis of FK-520 in recombinant form.

FK-520 is produced at relatively low levels in the naturally occurring cells, *Streptomyces hygroscopicus* var. *ascomyceticus*, in which it was first identified. Thus, another benefit provided by the recombinant FK-520 PKS and related genes of the present invention is the ability to produce FK-520 in greater quantities in the recombinant host cells provided by the invention. The invention also provides methods for making novel FK-520 analogs, in addition to the desmethoxy analogs described above, and derivatives in recombinant host cells of any origin.

The biosynthesis of FK-520 involves the action of several enzymes. The FK-520 PKS enzyme, which is composed of the *fkbA*, *fkbB*, *fkbC*, and *fkbP* gene products,

synthesizes the core structure of the molecule. There is also a hydroxylation at C-9 mediated by the P450 hydroxylase that is the *fkbD* gene product and that is oxidized by the *fkbO* gene product to result in the formation of a keto group at C-9. There is also a methylation at C-31 that is mediated by an O-methyltransferase that is the *fkbM* gene product. There are also methylations at the C-13 and C-15 positions by a methyltransferase believed to be encoded by the fkbG gene; this methyltransferase may act on the hydroxymalonyl CoA substrates prior to binding of the substrate to the AT domains of the PKS during polyketide synthesis. The present invention provides the genes encoding these enzymes in recombinant form. The invention also provides the genes encoding the enzymes involved in ethylmalonyl CoA and 2-hydroxymalonyl CoA biosynthesis in recombinant form. Moreover, the invention provides *Streptomyces hygroscopicus* var. *ascomyceticus* recombinant host cells lacking one or more of these genes that are useful in the production of useful compounds.

The cells are useful in production in a variety of ways. First, certain cells make a useful FK-520-related compound merely as a result of inactivation of one or more of the FK-520 biosynthesis genes. Thus, by inactivating the C-31 O-methyltransferase gene in *Streptomyces hygroscopicus* var. *ascomyceticus*, one creates a host cell that makes a desmethyl (at C-31) derivative of FK-520. Second, other cells of the invention are unable to make FK-520 or FK-520 related compounds due to an inactivation of one or more of the PKS genes. These cells are useful in the production of other polyketides produced by PKS enzymes that are encoded on recombinant expression vectors and introduced into the host cell.

Moreover, if only one PKS gene is inactivated, the ability to produce FK-520 or an FK-520 derivative compound is restored by introduction of a recombinant expression vector that contains the functional gene in a modified or unmodified form. The introduced gene produces a gene product that, together with the other endogenous and functional gene products, produces the desired compound. This methodology enables one to produce FK-520 derivative compounds without requiring that all of the genes for the PKS enzyme be present on one or more expression vectors. Additional applications and benefits of such cells and methodology will be readily apparent to those of skill in the art

after consideration of how the recombinant genes were isolated and employed in the construction of the compounds of the invention.

The FK-520 biosynthetic genes were isolated by the following procedure. Genomic DNA was isolated from *Streptomyces hygroscopicus* var. *ascomyceticus* (ATCC 14891) using the lysozyme/proteinase K protocol described in Genetic Manipulation of *Streptomyces* - A Laboratory Manual (Hopwood *et al.*, 1986). The average size of the DNA was estimated to be between 80 - 120 kb by electrophoresis on 0.3% agarose gels. A library was constructed in the SuperCos™ vector according to the manufacturer's instructions and with the reagents provided in the commercially available kit (Stratagene). Briefly, 100 µg of genomic DNA was partially digested with 4 units of *Sau*3A I for 20 min. in a reaction volume of 1 mL, and the fragments were dephosphorylated and ligated to SuperCos vector arms. The ligated DNA was packaged and used to infect log-stage XL1-BlueMR cells. A library of about 10,000 independent cosmid clones was obtained.

Based on recently published sequence from the FK-506 cluster (Motamedi and Shafiee, 1998, *Eur. J. Biochem. 256*: 528), a probe for the *fkbO* gene was isolated from ATCC 14891 using PCR with degenerate primers. With this probe, a cosmid designated pKOS034-124 was isolated from the library. With probes made from the ends of cosmid pKOS034-124, an additional cosmid designated pKOS034-120 was isolated. These cosmids (pKOS034-124 and pKOS034-120) were shown to contain DNA inserts that overlap with one another. Initial sequence data from these two cosmids generated sequences similar to sequences from the FK-506 and rapamycin clusters, indicating that the inserts were from the FK-520 PKS gene cluster. Two *Eco*RI fragments were subcloned from cosmids pKOS034-124 and pKOS034-120. These subclones were used to prepare shotgun libraries by partial digestion with *Sau*3AI, gel purification of fragments between 1.5 kb and 3 kb in size, and ligation into the pLitmus28 vector (New England Biolabs). These libraries were sequenced using dye terminators on a Beckmann CEQ2000 capillary electrophoresis sequencer, according to the manufacturer's protocols.

To obtain cosmids containing sequence on the left and right sides of the sequenced region described above, a new cosmid library of ATCC 14891 DNA was

prepared essentially as described above. This new library was screened with a new *fkbM* probe isolated using DNA from ATCC 14891. A probe representing the *fkbP* gene at the end of cosmid pKOS034-124 was also used. Several additional cosmids to the right of the previously sequenced region were identified. Cosmids pKOS065-C31 and pKOS065-C3 were identified and then mapped with restriction enzymes. Initial sequences from these cosmids were consistent with the expected organization of the cluster in this region. More extensive sequencing showed that both cosmids contained in addition to the desired sequences, other sequences not contiguous to the desired sequences on the host cell chromosomal DNA. Probing of additional cosmid libraries identified two additional cosmids, pKOS065-M27 and pKOS065-M21, that contained the desired sequences in a contiguous segment of chromosomal DNA. Cosmids pKOS034-124, pKOS034-120, pKOS065-M27, and pKOS065-M21 have been deposited with the American Type Culture Collection, Manassas, VA, USA. The complete nucleotide sequence of the coding sequences of the genes that encode the proteins of the FK-520 PKS are shown below but can also be determined from the cosmids of the invention deposited with the ATCC using standard methodology.

Referring to Figures 1 and 3, the FK-520 PKS gene cluster is composed of four open reading frames designated *fkbB*, *fkbC*, *fkbA*, and *fkbP*. The *fkbB* open reading frame encodes the loading module and the first four extender modules of the PKS. The *fkbC* open reading frame encodes extender modules five and six of the PKS. The *fkbA* open reading frame encodes extender modules seven, eight, nine, and ten of the PKS. The *fkbP* open reading frame encodes the NRPS of the PKS. Each of these genes can be isolated from the cosmids of the invention described above. The DNA sequences of these genes are provided below preceded by the following table identifying the start and stop codons of the open reading frames of each gene and the modules and domains contained therein.

Nucleotides	Gene or Domain
complement (412 - 1836)	*fkbW*
complement (2020 - 3579)	*fkbV*
complement (3969 - 4496)	*fkbR2*
complement (4595 - 5488)	*fkbR1*
5601 - 6818	*fkbE*

6808 - 8052	*fkbF*
8156 - 8824	*fkbG*
complement (9122 - 9883)	*fkbH*
complement (9894 - 10994)	*fkbI*
complement (10987 - 11247)	*fkbJ*
complement (11244 - 12092)	*fkbK*
complement (12113 - 13150)	*fkbL*
complement (13212 - 23988)	*fkbC*
complement (23992 - 46573)	*fkbB*
46754 - 47788	*fkbO*
47785 - 52272	*fkbP*
52275 - 71465	*fkbA*
71462 - 72628	*fkbD*
72625 - 73407	*fkbM*
complement (73460 - 76202)	*fkbN*
complement (76336 - 77080)	*fkbQ*
complement (77076 - 77535)	*fkbS*
complement (44974 - 46573)	CoA ligase of loading domain
complement (43777 - 44629)	ER of loading domain
complement (43144 - 43660)	ACP of loading domain
complement (41842 - 43093)	KS of extender module 1 (KS1)
complement(40609 - 41842)	AT1
complement (39442 - 40609)	DH1
complement (38677 - 39307)	KR1
complement (38371 - 38581)	ACP1
complement (37145 - 38296)	KS2
complement (35749 - 37144)	AT2
complement (34606 - 35749)	DH2 (inactive)
complement (33823 - 34480)	KR2
complement (33505 - 33715)	ACP2
complement (32185 - 33439)	KS3
complement (31018 - 32185)	AT3
complement (29869 - 31018)	DH3 (inactive)
complement (29092 - 29740)	KR3
complement (28750 - 28960)	ACP3
complement (27430 - 28684)	KS4
complement (26146 - 27430)	AT4
complement (24997 - 26146)	DH4 (inactive)
complement (24163 - 24373)	ACP4
complement (22653 - 23892)	KS5
complement (21420 - 22653)	AT5
complement (20241 - 21420)	DH5
complement (19464 - 20097)	KR5
complement (19116 - 19326)	ACP5

complement (17820 - 19053)	KS6
complement (16587 - 17820)	AT6
complement (15438 - 16587)	DH6
complement (14517 - 15294)	ER6
complement (13761 - 14394)	KR6
complement (13452 - 13662)	ACP6
52362 - 53576	KS7
53577 - 54716	AT7
54717 - 55871	DH7
56019 - 56819	ER7
56943 - 57575	KR7
57710 - 57920	ACP7
57990 - 59243	KS8
59244 - 60398	AT8
60399 - 61412	DH8 (inactive)
61548 - 62180	KR8
62328 - 62537	ACP8
62598 - 63854	KS9
63855 - 65084	AT9
65085 - 66254	DH9
66399 - 67175	ER9
67299 - 67931	KR9
68094 - 68303	ACP9
68397 - 69653	KS10
69654 - 70985	AT10
71064 - 71273	ACP10

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74941 CGCTCGATGG CGGCGGTGTC GACGCGCAGT GCGGCGTGGA CGGCGGGGTC GTCGGAGGCC
75001 CGGTAGGCGA ACTCCAGGTA GGTGACGGCC TCGTCGAGCT CGCCGCGCAG GTGGTGCTCG
75061 CGCGCGGCGT CGGTGAACAG CCCGGCGACC TCGGCGCCGT GCACCCGGCC GGTACCCATC
75121 TGGTGGCGGG CGAGCACCTT GCTGGCCACG CCGCGGTCCC GCAGCAGTTC CAGCGCCAGC
75181 TCGTGCAGGC CACGCCGCTC GGCGGCGGAG AGGTCGTCGA GTACGACGGA GCGGGCCGCG
75241 GGGTGCGGGA ACCGCCCTTC CCGCAGCAGC CGCCCCTCGA CCAGCTGTTC GTGGGCCTGC
75301 TCGACCGCCT CGGTGTCGAG GCCGGTCATC CGCTGGACGA GGGTGAGTTC GACACTCTCG
75361 CCGAGCACGG CGGAAGCTCG GGCGACGCTC AGCGCGGCCG GGCCGCAACG ATAGAGCGAC
75421 CCGAGGTAGG CGAGCCGGTA CGCCCGCCCC GCGACCACTT CCAGGCACCC TGAGGTCCGT
75481 GTCCGTGCCT CCCGGATGTC GTCGATCAGG CCGTGGCCGA GGAGCAGGTT GCCGCCGGTC
75541 GCCCGGAACG CCTGGGCCAC CACGTCGTCG TGCGCGTCCT GGCCGAGGTG CCGGCGCACG
75601 AGTTCGGTGG TCTGCGCCTC GGTGAGCGGG CGCAGCGCGA TCTCCTGGTA GTGGCGCAGA
75661 CTCAGCAGTG CCGCCCGGAA TTGGGAGTGG GCGGGCGTCG GCCGGAGCAG CTCGGTCAGC
75721 ACGATGGCGA CACGGGCCCG GCTGATGCGG CGCGCGAGGT GGAGCAGGCA GCGCAGCGAC
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75781 GGCGCGTCGG CGTGGTGCAC GTCGTCGATG CCGATCAGTA CGGGCCGCTC CGCGGCGAGC
75841 GTCAGCACCG TGCGGGTGAG TTCGGTCCCC AGGCGGTTGT CGACGTCGGC CGGCAGGTTT
75901 TCGCACGATG CCGTCAGCCG GACCAGCTCC GGTGTCCGGG CGGCCAGCTC GGGCTGGTCG
75961 AGGAGCTGGC CGAGCATGCC GTACGGCAGG GCCCGCTCCT CCATGGAGCA CACCGCGCGA
76021 AGGGTGACGA AGCCGGCCTT GGCCGCGGCG GCGTCGAGGA GTTCGGTCTT GCCGCAGGCG
76081 ATCGGCCCGG TGACGGCGGC GACGACGCCC CGCCCGCCCC CCGCTCGGGT GAGCGCCCGG
76141 TGGAGGGAAC CGAACTCGTC ATCGCGGGCG ATCAGGTCTG GGGGAGATAA GCGCGCTATC
76201 ACGAATGGAA CTACCTCGCG ACCGTCGTGG AAACCCATAG GCATCACATG GCTTGTTGAT
76261 CTGTACGGCT GTGATTCAGC CTGGCGGGAT GCTGTGCTAC AGATGGGAAG ATGTGATCTA
76321 GGGCCGTGCC GTTCCCTCAG GAGCCGACCG CCCCCGGCGC CACCCGCCGT ACCCCCTGGG
76381 CCACCAGCTC GGCGACCCGC TCCTGGTGGT CGACGAGGTA GAAGTGCCCG CCGGGGAAGA
76441 CCTCCACCGT GGTCGGCGCG GTCGTGTGCC CGGCCCAGGC GTGGGCCTGC TCCACCGTCG
76501 TCTTCGGATC GTCGTCACCG ATGCACACCG TGATCGGCGT CTCCAGCGGC GGCGCGGGCT
76561 CCCACCGGTA CGTCTCCGCC GCGTAGTAGT CCGCCCGCAA CGGCGCCAGG ATCAGCGCGC
76621 GCATTTCGTC GTCCGCCATC ACATCGGCGC TCGTCCCGCC GAGGCCGATG ACCGCCGCCA
76681 GCAGCTCGTC GTCGGACGCG AGGTGGTCCT GGTCGGCGCG CGGCTGCGAC GGCGCCCGCC
76741 GGCCCGAGAC GATCAGGTGC GCCACCGGGA GCCGCTGGGC CAGCTCGAAC GCGAGTGTCG
76801 CGCCCATGCT GTGGCCGAAC AGCACCAGCG GACGGTCCAG CCCCGGCTTC AACGCCTCGG
76861 CCACGAGGCC GGCGAGAACA CGCAGGTCGC GCACCGCCTC CTCGTCGCGG CGGTCCTGGC
76921 GGCCGGGGTA CTGCACGGCG TACACGTCCG CCACCGGGGC GAGCGCACGG GCCAGCGGAA
76981 GGTAGAACGT CGCCGATCCG CCGGCGTGGG GCAGCAGCAC CACCCGTACC GGGGCCTCGG
77041 GCGTGGGGAA GAACTGCCGC AGCCAGAGTT CCGAGCTCAC CGCACCCCCT CGGCCGCGAC
77101 CTGGGGAGCC CGGAACCGGG TGATCTCGGC CAAGTGCTTC TCCCGCATCT CCGGGTCGGT
77161 CACGCCCCAT CCCTCCTCCG GCGCCAGACA GAGGACGCCG ACTTTGCCGT TGTGCACATT
77221 GCGATGCACA TCGCGCACCG CCGACCCGAC GTCGTCGAGC GGGTAGGTCA CCGACAGCGT
77281 CGGGTGCACC ATCCCCTTGC AGATCAGGCG GTTCGCCTCC CACGCCTCAC GATAGTTCGC
77341 GAAGTGGGTA CCGATGATCC GCTTCACGGA CATCCACAGG TACCGATTGT CAAAGGCGTG
77401 CTCGTATCCC GAGGTTGACG CGCAGGTGAC GATCGTGCCA CCCCGACGTG TCACGTAGAC
77461 ACTCGCGCCG AACGTCGCGC GCCCCGGGTG CTCGAACACG ATGTCGGGAT CGTCACCGCC
77521 GGTCAGCTCC CGGATC
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Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the invention. The native DNA sequence encoding the FK-520 PKS of *Streptomyces hygroscopicus* is shown herein merely to illustrate a preferred embodiment of the invention, and the present invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the invention. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the amino acid sequences shown merely illustrate preferred embodiments of the invention.

The recombinant nucleic acids, proteins, and peptides of the invention are many and diverse. To facilitate an understanding of the invention and the diverse compounds and methods provided thereby, the following general description of the FK-520 PKS genes and modules of the PKS proteins encoded thereby is provided. This general description is followed by a more detailed description of the various domains and modules of the FK-520 PKS contained in and encoded by the compounds of the invention. In this description, reference to a heterologous PKS refers to any PKS other than the FK-520 PKS. Unless otherwise indicated, reference to a PKS includes reference to a portion of a PKS. Moreover, reference to a domain, module, or PKS includes reference to the nucleic acids encoding the same and vice-versa, because the methods and reagents of the invention provide or enable one to prepare proteins and the nucleic acids that encode them.

The FK-520 PKS is composed of three proteins encoded by three genes designated *fkbA*, *fkbB*, and *fkbC*. The *fkbA* ORF encodes extender modules 7 - 10 of the PKS. The *fkbB* ORF encodes the loading module (the CoA ligase) and extender modules 1 - 4 of the PKS. The *fkbC* ORF encodes extender modules 5 - 6 of the PKS. The *fkbP* ORF encodes the NRPS that attaches the pipecolic acid and cyclizes the FK-520 polyketide.

The loading module of the FK-520 PKS includes a CoA ligase, an ER domain, and an ACP domain. The starter building block or unit for FK-520 is believed to be a dihydroxycyclohexene carboxylic acid, which is derived from shikimate. The recombinant DNA compounds of the invention that encode the loading module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of methods and in a variety of compounds. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 loading module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for the loading module of the heterologous PKS is replaced by the coding sequence for the FK-520 loading module, provides a novel PKS coding sequence. Examples of heterologous PKS coding sequences include the rapamycin, FK-506, rifamycin, and avermectin PKS coding sequences. In another

embodiment, a DNA compound comprising a sequence that encodes the FK-520 loading module is inserted into a DNA compound that comprises the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the loading module coding sequence is utilized in conjunction with a heterologous coding sequence. In this embodiment, the invention provides, for example, either replacing the CoA ligase with a different CoA ligase, deleting the ER, or replacing the ER with a different ER. In addition, or alternatively, the ACP can be replaced by another ACP. In similar fashion, the corresponding domains in another loading or extender module can be replaced by one or more domains of the FK-520 PKS. The resulting heterologous loading module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide.

The first extender module of the FK-520 PKS includes a KS domain, an AT domain specific for methylmalonyl CoA, a DH domain, a KR domain, and an ACP domain. The recombinant DNA compounds of the invention that encode the first extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 first extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the first extender module of the FK-520 PKS or the latter is merely added to coding sequences for modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the first extender module of the FK-520 PKS is inserted into a DNA compound that comprises the remainder of the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or only a portion of the first extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-

hydroxymalonyl CoA specific AT; deleting either the DH or KR or both; replacing the DH or KR or both with another DH or KR; and/or inserting an ER. In replacing or inserting KR, DH, and ER domains, it is often beneficial to replace the existing KR, DH, and ER domains with the complete set of domains desired from another module. Thus, if one desires to insert an ER domain, one may simply replace the existing KR and DH domains with a KR, DH, and ER set of domains from a module containing such domains. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a gene for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous first extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the first extender module of the FK-520 PKS.

In an illustrative embodiment of this aspect of the invention, the invention provides recombinant PKSs and recombinant DNA compounds and vectors that encode such PKSs in which the KS domain of the first extender module has been inactivated. Such constructs are especially useful when placed in translational reading frame with the remaining modules and domains of an FK-520 or FK-520 derivative PKS. The utility of these constructs is that host cells expressing, or cell free extracts containing, the PKS encoded thereby can be fed or supplied with N-acylcysteamine thioesters of novel precursor molecules to prepare FK-520 derivatives. See U.S. patent application Serial No. 60/117,384, filed 27 Jan. 1999, and PCT patent publication Nos. US97/02358 and US99/03986, each of which is incorporated herein by reference.

The second extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the second extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes

the FK-520 second extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the second extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the second extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or a portion of the second extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR and/or the inactive DH; replacing the KR with another KR; and/or inserting an active DH or an active DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous second extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the second extender module of the FK-520 PKS.

The third extender module of the FK-520 PKS includes a KS, an AT specific for malonyl CoA, a KR, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the third extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 third extender module is inserted into a DNA compound that comprises the coding

sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the third extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the third extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or a portion of the third extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR and/or the inactive DH; replacing the KR with another KR; and/or inserting an active DH or an active DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous third extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the third extender module of the FK-520 PKS.

The fourth extender module of the FK-520 PKS includes a KS, an AT that binds ethylmalonyl CoA, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the fourth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 fourth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence

for a module of the heterologous PKS is either replaced by that for the fourth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the fourth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the remainder of the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the fourth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the ethylmalonyl CoA specific AT with a malonyl CoA, methylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; and/or deleting the inactive DH, inserting a KR, a KR and an active DH, or a KR, an active DH, and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, a PKS for a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous fourth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the fourth extender module of the FK-520 PKS.

As illustrative examples, the present invention provides recombinant genes, vectors, and host cells that result from the conversion of the FK-506 PKS to an FK-520 PKS and vice-versa. In one embodiment, the invention provides a recombinant set of FK-506 PKS genes but in which the coding sequences for the fourth extender module or at least those for the AT domain in the fourth extender module have been replaced by those for the AT domain of the fourth extender module of the FK-520 PKS. This recombinant PKS can be used to produce FK-520 in recombinant host cells. In another embodiment, the invention provides a recombinant set of FK-520 PKS genes but in which the coding sequences for the fourth extender module or at least those for the AT domain in the fourth

extender module have been replaced by those for the AT domain of the fourth extender module of the FK-506 PKS. This recombinant PKS can be used to produce FK-506 in recombinant host cells.

Other examples of hybrid PKS enzymes of the invention include those in which the AT domain of module 4 has been replaced with a malonyl specific AT domain to provide a PKS that produces 21-desethyl-FK520 or with a methylmalonyl specific AT domain to provide a PKS that produces 21-desethyl-21-methyl-FK520. Another hybrid PKS of the invention is prepared by replacing the AT and inactive KR domain of FK-520 extender module 4 with a methylmalonyl specific AT and an active KR domain, such as, for example, from module 2 of the DEBS or oleandolide PKS enzymes, to produce 21-desethyl-21-methyl-22-desoxo-22-hydroxy-FK520. The compounds produced by these hybrid PKS enzymes are neurotrophins.

The fifth extender module of the FK-520 PKS includes a KS, an AT that binds methylmalonyl CoA, a DH, a KR, and an ACP. The recombinant DNA compounds of the invention that encode the fifth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 fifth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fifth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fifth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the fifth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one or both of the DH and KR; replacing any one or both of the

DH and KR with either a KR and/or DH; and/or inserting an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous fifth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the fifth extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the DH domain of the fifth extender module have been deleted or mutated to render the DH non-functional. In one such mutated gene, the KR and DH coding sequences are replaced with those encoding only a KR domain from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-19 to C-20 double bond of FK-520 and has a C-20 hydroxyl group. Such analogs are preferred neurotrophins, because they have little or no immunosuppressant activity. This recombinant fifth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this fifth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (lacking the C-19 to C-20 double bond of FK-506 and having a C-20 hydroxyl group) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the DH domain of module 5 has been deleted or otherwise rendered inactive and thus produces this novel polyketide.

The sixth extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the sixth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 sixth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the sixth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the sixth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the sixth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the KR, DH, and ER; and/or replacing any one, two, or all three of the KR, DH, and ER with another KR, DH, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous sixth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the sixth extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the DH and ER domains of the sixth extender module have been deleted or mutated to render them non-functional. In one such mutated gene, the KR, ER, and DH coding sequences are replaced with those encoding only a KR domain from another PKS gene. This can also be accomplished by simply replacing the coding sequences for extender module six with those for an extender module having a methylmalonyl specific AT and only a KR domain from a heterologous PKS gene, such as, for example, the coding sequences for extender module two encoded by the *eryAI* gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that has a C-18 hydroxyl group. Such analogs are preferred neurotrophins, because they have little or no immunosuppressant activity. This recombinant sixth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this sixth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (having a C-18 hydroxyl group) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the DH and ER domains of module 6 have been deleted or otherwise rendered inactive and thus produces this novel polyketide.

The seventh extender module of the FK-520 PKS includes a KS, an AT specific for 2-hydroxymalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the seventh extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 seventh extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the seventh

extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the seventh extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion or all of the seventh extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the 2-hydroxymalonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or malonyl CoA specific AT; deleting the KR, the DH, and/or the ER; and/or replacing the KR, DH, and/or ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous seventh extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the seventh extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the AT domain of the seventh extender module has been replaced with those encoding an AT domain for malonyl, methylmalonyl, or ethylmalonyl CoA from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-15 methoxy group, having instead a hydrogen, methyl, or ethyl group at that position, respectively. Such analogs are preferred, because they are more slowly metabolized than FK-520. This recombinant seventh extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that

contains both this seventh extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (C-15-desmethoxy) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the AT domain of module 7 has been replaced and thus produces this novel polyketide.

In another illustrative embodiment, the present invention provides a hybrid PKS in which the AT and KR domains of module 7 of the FK-520 PKS are replaced by a methylmalonyl specific AT domain and an inactive KR domain, such as, for example, the AT and KR domains of extender module 6 of the rapamycin PKS. The resulting hybrid PKS produces 15-desmethoxy-15-methyl-16-oxo-FK-520, a neurotrophin compound.

The eighth extender module of the FK-520 PKS includes a KS, an AT specific for 2-hydroxymalonyl CoA, a KR, and an ACP. The recombinant DNA compounds of the invention that encode the eighth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 eighth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the eighth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the eighth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the eighth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the 2-

hydroxymalonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or malonyl CoA specific AT; deleting or replacing the KR; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous eighth extender module coding sequence can be utilized in conjunction with a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the eighth extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the AT domain of the eighth extender module has been replaced with those encoding an AT domain for malonyl, methylmalonyl, or ethylmalonyl CoA from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-13 methoxy group, having instead a hydrogen, methyl, or ethyl group at that position, respectively. Such analogs are preferred, because they are more slowly metabolized than FK-520. This recombinant eighth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this eighth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (C-13-desmethoxy) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the AT domain of module 8 has been replaced and thus produces this novel polyketide.

The ninth extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the ninth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 ninth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the ninth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the ninth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the ninth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the KR, DH, and ER; and/or replacing any one, two, or all three of the KR, DH, and ER with another KR, DH, and/or ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous ninth extender module coding sequence can be utilized in conjunction with a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the ninth extender module of the FK-520 PKS.

The tenth extender module of the FK-520 PKS includes a KS, an AT specific for malonyl CoA, and an ACP. The recombinant DNA compounds of the invention that encode the tenth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 tenth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the tenth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the tenth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion or all of the tenth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; and/or inserting a KR, a KR and DH, or a KR, DH, and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous tenth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the tenth extender module of the FK-520 PKS.

The FK-520 polyketide precursor produced by the action of the tenth extender module of the PKS is then attached to pipecolic acid and cyclized to form FK-520. The

enzyme FkbP is the NRPS like enzyme that catalyzes these reactions. FkbP also includes a thioesterase activity that cleaves the nascent FK-520 polyketide from the NRPS. The present invention provides recombinant DNA compounds that encode the *fkbP* gene and so provides recombinant methods for expressing the *fkbP* gene product in recombinant host cells. The recombinant *fkbP* genes of the invention include those in which the coding sequence for the adenylation domain has been mutated or replaced with coding sequences from other NRPS like enzymes so that the resulting recombinant FkbP incorporates a moiety other than pipecolic acid. For the construction of host cells that do not naturally produce pipecolic acid, the present invention provides recombinant DNA compounds that express the enzymes that catalyze at least some of the biosynthesis of pipecolic acid (see Nielsen *et al.*, 1991, *Biochem. 30*: 5789-96). The *fkbL* gene encodes a homolog of RapL, a lysine cyclodeaminase responsible in part for producing the pipecolate unit added to the end of the polyketide chain. The *fkbB* and *fkbL* recombinant genes of the invention can be used in heterologous hosts to produce compounds such as FK-520 or, in conjunction with other PKS or NRPS genes, to produce known or novel polyketides and non-ribosmal peptides.

The present invention also provides recombinant DNA compounds that encode the P450 oxidase and methyltransferase genes involved in the biosynthesis of FK-520. Figure 2 shows the various sites on the FK-520 polyketide core structure at which these enzymes act. By providing these genes in recombinant form, the present invention provides recombinant host cells that can produce FK-520. This is accomplished by introducing the recombinant PKS, P450 oxidase, and methyltransferase genes into a heterologous host cell. In a preferred embodiment, the heterologous host cell is *Streptomyces coelicolor* CH999 or *Streptomyces lividans* K4-114, as described in U.S. Patent No. 5,830,750 and U.S. patent application Serial Nos. 08/828,898, filed 31 Mar. 1997, and 09/181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference. In addition, by providing recombinant host cells that express only a subset of these genes, the present invention provides methods for making FK-520 precursor compounds not readily obtainable by other means.

In a related aspect, the present invention provides recombinant DNA compounds and vectors that are useful in generating, by homologous recombination, recombinant host cells that produce FK-520 precursor compounds. In this aspect of the invention, a native host cell that produces FK-520 is transformed with a vector (such as an SCP2* derived vector for *Streptomyces* host cells) that encodes one or more disrupted genes (i.e., a hydroxylase, a methyltransferase, or both) or merely flanking regions from those genes. When the vector integrates by homologous recombination, the native, functional gene is deleted or replaced by the non-functional recombinant gene, and the resulting host cell thus produces an FK-520 precursor. Such host cells can also be complemented by introduction of a modified form of the deleted or mutated non-functional gene to produce a novel compound.

In one important embodiment, the present invention provides a hybrid PKS and the corresponding recombinant DNA compounds that encode those hybrid PKS enzymes. For purposes of the present invention a hybrid PKS is a recombinant PKS that comprises all or part of one or more modules and thioesterase/cyclase domain of a first PKS and all or part of one or more modules, loading module, and thioesterase/cyclase domain of a second PKS. In one preferred embodiment, the first PKS is all or part of the FK-520 PKS, and the second PKS is only a portion or all of a non-FK-520 PKS.

One example of the preferred embodiment is an FK-520 PKS in which the AT domain of module 8, which specifies a hydroxymalonyl CoA and from which the C-13 methoxy group of FK-520 is derived, is replaced by an AT domain that specifies a malonyl, methylmalonyl, or ethylmalonyl CoA. Examples of such replacement AT domains include the AT domains from modules 3, 12, and 13 of the rapaymycin PKS and from modules 1 and 2 of the erythromycin PKS. Such replacements, conducted at the level of the gene for the PKS, are illustrated in the examples below. Another illustrative example of such a hybrid PKS includes an FK-520 PKS in which the natural loading module has been replaced with a loading module of another PKS. Another example of such a hybrid PKS is an FK-520 PKS in which the AT domain of module three is replaced with an AT domain that binds methylmalonyl CoA.

In another preferred embodiment, the first PKS is most but not all of a non-FK-520 PKS, and the second PKS is only a portion or all of the FK-520 PKS. An illustrative example of such a hybrid PKS includes an erythromycin PKS in which an AT specific for methylmalonyl CoA is replaced with an AT from the FK-520 PKS specfic for malonyl CoA.

Those of skill in the art will recognize that all or part of either the first or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See U.S. provisional patent application Serial No. 60/091,526, incorporated herein by reference. The state of the art in DNA synthesis allows the artisan to construct *de novo* DNA compounds of size sufficient to construct a useful portion of a PKS module or domain. For purposes of the present invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

Thus, the hybrid modules of the invention are incorporated into a PKS to provide a hybrid PKS of the invention. A hybrid PKS of the invention can result not only:

(i) from fusions of heterologous domain (where heterologous means the domains in that module are from at least two different naturally occurring modules) coding sequences to produce a hybrid module coding sequence contained in a PKS gene whose product is incorporated into a PKS,

but also:

(ii) from fusions of heterologous module (where heterologous module means two modules are adjacent to one another that are not adjacent to one another in naturally occurring PKS enzymes) coding sequences to produce a hybrid coding sequence contained in a PKS gene whose product is incorporated into a PKS,

(iii) from expression of one or more FK-520 PKS genes with one or more non-FK-520 PKS genes, including both naturally occurring and recombinant non-FK-520 PKS genes, and

(iv) from combinations of the foregoing.

Various hybrid PKSs of the invention illustrating these various alternatives are described herein.

Examples of the production of a hybrid PKS by co-expression of PKS genes from the FK-520 PKS and another non-FK-520 PKS include hybrid PKS enzymes produced by coexpression of FK-520 and rapamycin PKS genes. Preferably, such hybrid PKS enzymes are produced in recombinant *Streptomyces* host cells that produce FK-520 or FK-506 but have been mutated to inactivate the gene whose function is to be replaced by the rapamycin PKS gene introduced to produce the hybrid PKS. Particular examples include (i) replacement of the *fkbC* gene with the *rapB* gene; and (ii) replacement of the *fkbA* gene with the *rapC* gene. The latter hybrid PKS produces 13,15-didesmethoxy-FK-520, if the host cell is an FK-520 producing host cell, and 13,15-didesmethoxy-FK-506, if the host cell is an FK-506 producing host cell. The compounds produced by these hybrid PKS enzymes are immunosuppressants and neurotrophins but can be readily modified to act only as neurotrophins, as described in Example 6, below.

Other illustrative hybrid PKS enzymes of the invention are prepared by replacing the *fkbA* gene of an FK-520 or FK-506 producing host cell with a hybrid *fkbA* gene in which: (a) the extender module 8 through 10, inclusive, coding sequences have been replaced by the coding sequnces for extender modules 12 to 14, inclusive, of the rapamycin PKS; and (b) the module 8 coding sequences have been replaced by the module 8 coding sequence of the rifamycin PKS. When expressed with the other, naturally occurring FK-520 or FK-506 PKS genes and the genes of the modification enzymes, the resulting hybrid PKS enzymes produce, respectively, (a) 13-desmethoxy-FK-520 or 13-desmethoxy-FK-506; and (b) 13-desmethoxy-13-methyl-FK-520 or 13-desmethoxy-13-methyl-FK-506. In a preferred embodiment, these recombinant PKS genes of the invention are introduced into the producing host cell by a vector such as pHU204, which is a plamsid pRM5 derivative that has the well-characterized SCP2* replicon, the *colE1* replicon, the *tsr* and *bla* resistance genes, and a *cos* site. This vector can be used to introduce the recombinant *fkbA* replacement gene in an FK-520 or FK-506 producing host cell (or a host cell derived therefrom in which the endogenous fkbA gene has either been rendered inactive by mutation, deletion or homologous recombination with the gene that replaces it) to produce the desired hybrid PKS.

In constructing hybrid PKSs of the invention, certain general methods may be helpful. For example, it is often beneficial to retain the framework of the module to be altered to make the hybrid PKS. Thus, if one desires to add DH and ER functionalities to a module, it is often preferred to replace the KR domain of the original module with a KR, DH, and ER domain-containing segment from another module, instead of merely inserting DH and ER domains. One can alter the stereochemical specificity of a module by replacement of the KS domain with a KS domain from a module that specifies a different stereochemistry. See Lau *et al.*, 1999, "Dissecting the role of acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units," *Biochemistry 38*(5):1643-1651, incorporated herein by reference. Stereochemistry can also be changed by changing the KR domain. Also, one can alter the specificity of an AT domain by changing only a small segment of the domain. See Lau *et al., supra*. One can also take advantage of known linker regions in PKS proteins to link modules from two different PKSs to create a hybrid PKS. See Gokhale *et al.*, 16 Apr. 1999, "Dissecting and Exploiting Intermodular Communication in Polyketide Synthases," *Science 284*: 482-485, incorporated herein by reference.

The following Table lists references describing illustrative PKS genes and corresponding enzymes that can be utilized in the construction of the recombinant PKSs and the corresponding DNA compounds that encode them of the invention. Also presented are various references describing tailoring enzymes and corresponding genes that can be employed in accordance with the methods of the present invention.

Avermectin

U.S. Pat. No. 5,252,474 to Merck.

MacNeil *et al.*, 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.

MacNeil *et al.*, 1992, *Gene 115*: 119-125, Complex Organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase.

Ikeda *et al.*, Aug. 1999, Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*, *Proc. Natl. Acad. Sci. USA 96*: 9509-9514.

Candicidin (FR008)

Hu *et al.*, 1994, *Mol. Microbiol.* 14: 163-172.

Epothilone

U.S. Pat. App. Serial No. 60/130,560, filed 22 April 1999.

Erythromycin

PCT Pub. No. 93/13663 to Abbott.

US Pat. No. 5,824,513 to Abbott.

Donadio *et al.*, 1991, *Science 252*:675-9.

Cortes *et al.*, 8 Nov. 1990, *Nature 348*:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of *Saccharopolyspora erythraea*.

<u>Glycosylation Enzymes</u>

PCT Pat. App. Pub. No. 97/23630 to Abbott.

FK-506

Motamedi *et al.*, 1998, The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506, *Eur. J. biochem. 256*: 528-534.

Motamedi *et al.*, 1997, Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506, *Eur. J. Biochem. 244*: 74-80.

<u>Methyltransferase</u>

US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858. 31-O-desmethyl-FK-506 methyltransferase.

Motamedi *et al.*, 1996, Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK-506 and FK-520, *J. Bacteriol. 178*: 5243-5248.

Streptomyces hygroscopicus

U.S. patent application Serial No. 09/154,083, filed 16 Sep. 1998.

Lovastatin

U.S. Pat. No. 5,744,350 to Merck.

Narbomycin

U.S. patent application Serial No. 60/107,093, filed 5 Nov. 1998, and Serial No. 60/120,254, filed 16 Feb. 1999.

Nemadectin

MacNeil *et al.*, 1993, *supra.*

Niddamycin

Kakavas *et al.*, 1997, Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*, *J. Bacteriol. 179*: 7515-7522.

Oleandomycin

Swan *et al.*, 1994, Characterisation of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, *Mol. Gen. Genet. 242*: 358-362.

U.S. patent application Serial No. 60/120,254, filed 16 Feb. 1999.

Olano *et al.*, 1998, Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring, *Mol. Gen. Genet. 259*(3): 299-308.

Picromycin

PCT patent application US99/15047, filed 2 Jul. 1999.

Xue *et al.*, 1998, Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the *pik*C-encoded cytochrome P450 in *Streptomyces venezuelae*, *Chemistry & Biology 5*(11): 661-667.

Xue *et al.*, Oct. 1998, A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity, *Proc. Natl. Acad. Sci. USA 95*: 12111 12116.

Platenolide

EP Pat. App. Pub. No. 791,656 to Lilly.

Rapamycin

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, *Proc. Natl. Acad. Sci. USA 92*:7839-7843.

Aparicio *et al.*, 1996, Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase, *Gene 169*: 9-16.

Rifamycin

August *et al.*, 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S669, *Chemistry & Biology, 5*(2): 69-79.

***Sorangium* PKS**

U.S. patent application Serial No. 09/144,085, filed 31 Aug. 1998.

Soraphen

U.S. Pat. No. 5,716,849 to Novartis.

Schupp *et al.*, 1995, *J. Bacteriology 177*: 3673-3679. A *Sorangium cellulosum* (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

Spiramycin

U.S. Pat. No. 5,098,837 to Lilly.

Activator Gene

U.S. Pat. No. 5,514,544 to Lilly.

Tylosin

EP Pub. No. 791,655 to Lilly.

U.S. Pat. No. 5,876,991 to Lilly.

Kuhstoss *et al.*, 1996, *Gene 183*:231-6., Production of a novel polyketide through the construction of a hybrid polyketide synthase.

Tailoring enzymes

Merson-Davies and Cundliffe, 1994, *Mol. Microbiol. 13*: 349-355. Analysis of five tylosin biosynthetic genes from the *tylBA* region of the *Streptomyces fradiae* genome.

As the above Table illustrates, there are a wide variety of polyketide synthase genes that serve as readily available sources of DNA and sequence information for use in constructing the hybrid PKS-encoding DNA compounds of the invention. Methods for constructing hybrid PKS-encoding DNA compounds are described without reference to the FK-520 PKS in PCT patent publication No. 98/51695; U.S. Patent Nos. 5,672,491 and 5,712,146 and U.S. patent application Serial Nos. 09/073,538, filed 6 May 1998, and 09/141,908, filed 28 Aug 1998, each of which is incorporated herein by reference.

The hybrid PKS-encoding DNA compounds of the invention can be and often are hybrids of more than two PKS genes. Moreover, there are often two or more modules in the hybrid PKS in which all or part of the module is derived from a second (or third) PKS. Thus, as one illustrative example, the present invention provides a hybrid FK-520 PKS that contains the naturally occurring loading module and FkbP as well as modules one, two, four, six, seven, and eight, nine, and ten of the FK-520 PKS and further contains hybrid or heterologous modules three and five. Hybrid or heterologous module three contains an AT domain that is specific of methylmalonyl CoA and can be derived for example, from the erythromycin or rapamycin PKS genes. Hybrid or heterologous module five contains an AT domain that is specific for malonyl CoA and can be derived for example, from the picromycin or rapamycin PKS genes.

While an important embodiment of the present invention relates to hybrid PKS enzymes and corresponding genes, the present invention also provides recombinant FK-520 PKS genes in which there is no second PKS gene sequence present but which differ from the FK-520 PKS gene by one or more deletions. The deletions can encompass one or more modules and/or can be limited to a partial deletion within one or more modules. When a deletion encompasses an entire module, the resulting FK-520 derivative is at least two carbons shorter than the gene from which it was derived. When a deletion is within a module, the deletion typically encompasses a KR, DH, or ER domain, or both

DH and ER domains, or both KR and DH domains, or all three KR, DH, and ER domains.

To construct a hybrid PKS or FK-520 derivative PKS gene of the invention, one can employ a technique, described in PCT Pub. No. 98/27203 and U.S. patent application Serial No. 08/989,332, filed 11 Dec. 1997, each of which is incorporated herein by reference, in which the large PKS gene is divided into two or more, typically three, segments, and each segment is placed on a separate expression vector. In this manner, each of the segments of the gene can be altered, and various altered segments can be combined in a single host cell to provide a recombinant PKS gene of the invention. This technique makes more efficient the construction of large libraries of recombinant PKS genes, vectors for expressing those genes, and host cells comprising those vectors.

Thus, in one important embodiment, the recombinant DNA compounds of the invention are expression vectors. As used herein, the term expression vector refers to any nucleic acid that can be introduced into a host cell or cell-free transcription and translation medium. An expression vector can be maintained stably or transiently in a cell, whether as part of the chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a gene that serves to produce RNA that is translated into a polypeptide in the cell or cell extract. Furthermore, expression vectors typically contain additional functional elements, such as resistance-conferring genes to act as selectable markers.

The various components of an expression vector can vary widely, depending on the intended use of the vector. In particular, the components depend on the host cell(s) in which the vector will be used or is intended to function. Vector components for expression and maintenance of vectors in *E. coli* are widely known and commercially available, as are vector components for other commonly used organisms, such as yeast cells and *Streptomyces* cells.

In a preferred embodiment, the expression vectors of the invention are used to construct recombinant *Streptomyces* host cells that express a recombinant PKS of the invention. Preferred *Streptomyces* host cell/vector combinations of the invention include *S. coelicolor* CH999 and *S. lividans* K4-114 host cells, which do not produce

actinorhodin, and expression vectors derived from the pRM1 and pRM5 vectors, as described in U.S. Patent No. 5,830,750 and U.S. patent application Serial Nos. 08/828,898, filed 31 Mar. 1997, and 09/181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference.

The present invention provides a wide variety of expression vectors for use in *Streptomyces*. For replicating vectors, the origin of replication can be, for example and without limitation, a low copy number vector, such as SCP2* (see Hopwood *et al.*, *Genetic Manipulation of Streptomyces: A Laboratory manual* (The John Innes Foundation, Norwich, U.K., 1985); Lydiate *et al.*, 1985, *Gene 35*: 223-235; and Kieser and Melton, 1988, *Gene 65*: 83-91, each of which is incorporated herein by reference), SLP1.2 (Thompson *et al.*, 1982, *Gene 20*: 51-62, incorporated herein by reference), and SG5(ts) (Muth *et al.*, 1989, *Mol. Gen. Genet. 219*: 341-348, and Bierman *et al.*, 1992, *Gene 116*: 43-49, each of which is incorporated herein by reference), or a high copy number vector, such as pIJ101 and pJV1 (see Katz *et al.*, 1983, *J. Gen. Microbiol. 129*: 2703-2714; Vara *et al.*, 1989, *J. Bacteriol. 171*: 5782-5781; and Servin-Gonzalez, 1993, *Plasmid 30*: 131-140, each of which is incorporated herein by reference). Generally, however, high copy number vectors are not preferred for expression of genes contained on large segments of DNA. For non-replicating and integrating vectors, it is useful to include at least an *E. coli* origin of replication, such as from pUC, p1P, p1I, and pBR. For phage based vectors, the phages phiC31 and KC515 can be employed (see Hopwood *et al.*, *supra*).

Typically, the expression vector will comprise one or more marker genes by which host cells containing the vector can be identified and/or selected. Useful antibiotic resistance conferring genes for use in *Streptomyces* host cells include the *ermE* (confers resistance to erythromycin and other macrolides and lincomycin), *tsr* (confers resistance to thiostrepton), *aadA* (confers resistance to spectinomycin and streptomycin), *aacC4* (confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418), and neomycin), *hyg* (confers resistance to hygromycin), and *vph* (confers resistance to viomycin) resistance conferring genes.

The recombinant PKS gene on the vector will be under the control of a promoter, typically with an attendant ribosome binding site sequence. The present invention provides the endogenous promoters of the FK-520 PKS and related biosynthetic genes in recombinant form, and these promoters are preferred for use in the native hosts and in heterologous hosts in which the promoters function. A preferred promoter of the invention is the *fkbO* gene promoter, comprised in a sequence of about 270 bp between the start of the open reading frames of the *fkbO* and *fkbB* genes. The *fkbO* promoter is believed to be bi-directional in that it promotes transcription of the genes *fkbO*, *fkbP*, and *fkbA* in one direction and *fkbB*, *fkbC*, and *fkbL* in the other. Thus, in one aspect, the present invention provides a recombinant expression vector comprising the promoter of the *fkbO* gene of an FK-520 producing organism positioned to transcribe a gene other than *fkbO*. In a preferred embodiment the transcribed gene is an FK-520 PKS gene. In another preferred embodiment, the transcribed gene is a gene that encodes a protein comprised in a hybrid PKS.

Heterologous promoters can also be employed and are preferred for use in host cells in which the endogenous FK-520 PKS gene promoters do not function or function poorly. A preferred heterologous promoter is the *actI* promoter and its attendant activator gene *actII-ORF4*, which is provided in the pRM1 and pRM5 expression vectors, *supra*. This promoter is activated in the stationary phase of growth when secondary metabolites are normally synthesized. Other useful *Streptomyces* promoters include without limitation those from the *ermE* gene and the *melC1* gene, which act constitutively, and the *tipA* gene and the *merA* gene, which can be induced at any growth stage. In addition, the T7 RNA polymerase system has been transferred to *Streptomyces* and can be employed in the vectors and host cells of the invention. In this system, the coding sequence for the T7 RNA polymerase is inserted into a neutral site of the chromosome or in a vector under the control of the inducible *merA* promoter, and the gene of interest is placed under the control of the T7 promoter. As noted above, one or more activator genes can also be employed to enhance the activity of a promoter. Activator genes in addition to the *actII-ORF4* gene discussed above include *dnrI*, *redD*, and *ptpA* genes (see U.S. patent application Serial No. 09/181,833, *supra*) to activate promoters under their control.

In addition to providing recombinant DNA compounds that encode the FK-520 PKS, the present invention also provides DNA compounds that encode the ethylmalonyl CoA and 2-hydroxymalonyl CoA utilized in the synthesis of FK-520. Thus, the present invention also provides recombinant host cells that express the genes required for the biosynthesis of ethylmalonyl CoA and 2-hydroxymalonyl CoA. Figures 3 and 4 show the location of these genes on the cosmids of the invention and the biosynthetic pathway that produces ethylmalonyl CoA.

For 2-hydroxymalonyl CoA biosynthesis, the *fkbH*, *fkbI*, *fkbJ*, and *fkbK* genes are sufficient to confer this ability on *Streptomcyces* host cells. For conversion of 2-hydroxymalonyl to 2-methoxymalonyl, the *fkbG* gene is also employed. While the complete coding sequence for *fkbH* is provided on the cosmids of the invention, the sequence for this gene provided herein may be missing a T residue, based on a comparison made with a similar gene cloned from the ansamitocin gene cluster by Dr. H. Floss. Where the sequence herein shows one T, there may be two, resulting in an extension of the *fkbH* reading frame to encode the amino acid sequence: MTIVKCLVWDLDNTLWRGTVLEDDEVVLTDEIREVITTLDDRGILQAVASKNDHDLAWERLERLGVAEYFVLARIGWGPKSQSVREIATELNFAPTTIAFIDDQPAERAEVAFHLPEVRCYPAEQAATLLSLPEFSPPVSTVDSRRRRLMYQAGFARDQAREAYSGPDEDFLRSLDLSMTIAPAGEEELSRVEELTLRTSQMNATGVHYSDADLRALLTDPAHEVLVVTMGDRFGPHGAVGIILLEKKPSTWHLKLLATSCRVVSFGAGATILNWLTDQGARAGAHLVADFRRTDRNRMMEIAYRFAGFADSDCPCVSEVAGASAAGVERLHLEPSARPAPTTLTLTAADIAPVTVSAAG.

For ethylmalonyl CoA biosynthesis, one requires only a crotonyl CoA reductase, which can be supplied by the host cell but can also be supplied by recombinant expression of the *fkbS* gene of the present invention. To increase yield of ethylmalonyl CoA, one can also express the *fkbE* and *fkbU* genes as well. While such production can be achieved using only the recombinant genes above, one can also achieve such production by placing into the recombinant host cell a large segment of the DNA provided by the cosmids of the invention. Thus, for 2-hydroxymalonyl and 2-methoxymalonyl CoA biosynthesis, one can simply provide the cells with the segment of

DNA located on the left side of the FK-520 PKS genes shown in Figure 1. For ethylmalonyl CoA biosynthesis, one can simply provide the cells with the segment of DNA located on the right side of the FK-520 PKS genes shown in Figure 1 or, alternatively, both the right and left segments of DNA.

The recombinant DNA expression vectors that encode these genes can be used to construct recombinant host cells that can make these important polyketide building blocks from cells that otherwise are unable to produce them. For example, *Streptomyces coelicolor* and *Streptomyces lividans* do not synthesisze ethylmalonyl CoA or 2-hydroxymalonyl CoA. The invention provides methods and vectors for constructing recombinant *Streptomyces coelicolor* and *Streptomyces lividans* that are able to synthesize either or both ethylmalonyl CoA and 2-hydroxymalonyl CoA. These host cells are thus able to make polyketides, those requiring these substrates, that cannot otherwise be made in such cells.

In a preferred embodiment, the present invention provides recombinant *Streptomyces* host cells, such as *S. coelicolor* and *S. lividans*, that have been transformed with a recombinant vector of the invention that codes for the expression of the ethylmalonyl CoA biosynthetic genes. The resulting host cells produce ethylmalonyl CoA and so are preferred host cells for the production of polyketides produced by PKS enzymes that comprise one or more AT domains specific for ethylmalonyl CoA. Illustrative PKS enzymes of this type include the FK-520 PKS and a recombinant PKS in which one or more AT domains is specific for ethylmalonyl CoA.

In a related embodiment, the present invention provides *Streptomyces* host cells in which one or more of the ethylmalonyl or 2-hydroxymalonyl biosynthetic genes have been deleted by homologous recombination or rendered inactive by mutation. For example, deletion or inactivation of the *fkbG* gene can prevent formation of the methoxyl groups at C-13 and C-15 of FK-520 (or, in the corresponding FK-506 producing cell, FK-506), leading to the production of 13,15-didesmethoxy-13,15-dihydroxy-FK-520 (or, in the corresponding FK-506 producing cell, 13,15-didesmethoxy-13,15-dihydroxy-FK-506). If the *fkbG* gene product acts on 2-hydroxymalonyl and the resulting 2-methoxymalonyl substrate is required for incorporation by the PKS, the AT domains of

modules 7 and 8 may bind malonyl CoA and methylmalonyl CoA. Such incorporation results in the production of a mixture of polyketides in which the methoxy groups at C-13 and C-15 of FK-520 (or FK-506) are replaced by either hydrogen or methyl.

This possibility of non-specific binding results from the construction of a hybrid PKS of the invention in which the AT domain of module 8 of the FK-520 PKS replaced the AT domain of module 6 of DEBS. The resulting PKS produced, in *Streptomyces lividans*, 6-dEB and 2-desmethyl-6-dEB, indicating that the AT domain of module 8 of the FK-520 PKS could bind malonyl CoA and methylmalonyl CoA substrates. Thus, one could possibly also prepare the 13,15-didesmethoxy-FK-520 and corresponding FK-506 compounds of the invention by deleting or otherwise inactivating one or more or all of the genes required for 2-hydroxymalonyl CoA biosynthesis, i.e., the *fkbH*, *fkbI*, *fkbJ*, and *fkbK* genes. In any event, the deletion or inactivation of one or more biosynthetic genes required for ethylmalonyl and/or 2-hydroxymalonyl production prevents the formation of polyketides requiring ethylmalonyl and/or 2-hydroxymalonyl for biosynthesis, and the resulting host cells are thus preferred for production of polyketides that do not require the same.

The host cells of the invention can be grown and fermented under conditions known in the art for other purposes to produce the compounds of the invention. See, e.g., U.S. Patent Nos. 5,194,378; 5,116,756; and 5,494,820, incorporated herein by reference, for suitable fermentation processes. The compounds of the invention can be isolated from the fermentation broths of these cultured cells and purified by standard procedures. Preferred compounds of the invention include the following compounds: 13-desmethoxy-FK-506; 13-desmethoxy-FK-520; 13,15-didesmethoxy-FK-506; 13,15-didesmethoxy-FK-520; 13-desmethoxy-18-hydroxy-FK-506; 13-desmethoxy-18-hydroxy-FK-520; 13,15-didesmethoxy-18-hydroxy-FK-506; and 13,15-didesmethoxy-18-hydroxy-FK-520. These compounds can be further modified as described for tacrolimus and FK-520 in U.S. Patent Nos. 5,225,403; 5,189,042; 5,164,495; 5,068,323; 4,980,466; and 4,920,218, incorporated herein by reference.

Other compounds of the invention are shown in Figure 8, Parts A and B. In Figure 8, Part A, illustrative C-32-substituted compounds of the invention are shown in two

columns under the heading R. The substituted compounds are preferred for topical administration and are applied to the dermis for treatment of conditions such as psoriasis. In Figure 8, Part B, illustrative reaction schemes for making the compounds shown in Figure 8, Part A, are provided. In the upper scheme in Figure 8, Part B, the C-32 substitution is a tetrazole moiety, illustrative of the groups shown in the left column under R in Figure 8, Part A. In the lower scheme in Figure 8, Part B, the C-32 substitution is a disubstituted amino group, where R_3 and R_4 can be any group similar to the illustrative groups shown attached to the amine in the right column under R in Figure 8, Part A. While Figure 8 shows the C-32-substituted compounds in which the C-15-methoxy is present, the invention includes these C-32-substituted compounds in which C-15 is ethyl, methyl, or hydrogen. Also, while C-21 is shown as substituted with ethyl or allyl, the compounds of the invention includes the C-32-substituted compounds in which C-21 is substituted with hydrogen or methyl.

To make these C-32-substituted compounds, Figure 8, Part B, provides illustrative reaction schemes. Thus, a selective reaction of the starting compound (see Figure 8, Part B, for an illustrative starting compound) with trifluoromethanesulfonic anhydride in the presence of a base yields the C-32 O-triflate derivative, as shown in the upper scheme of Figure 8, Part B. Displacement of the triflate with 1H-tetrazole or triazole derivatives provides the C-32 tetrazole or teiazole derivative. As shown in the lower scheme of Figure 8, Part B, reacting the starting compound with p-nitrophenylchloroformate yields the correspoinding carbonate, which, upon displacement with an amino compound, provides the corresponding carbamate derivative.

The compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation contains one or more of the compounds of the invention as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any

other form suitable for use. Suitable formulation processes and compositions for the compounds of the present invention are described with respect to tacrolimus in U.S. Patent Nos. 5,939,427; 5,922,729; 5,385,907; 5,338,684; and 5,260,301, incorporated herein by reference. Many of the compounds of the invention contain one or more chiral centers, and all of the stereoisomers are included within the scope of the invention, as pure compounds as well as mixtures of stereoisomers. Thus the compounds of the invention may be supplied as a mixture of stereoisomers in any proportion.

The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo *et al.*, 1987, *Transplantation Proceedings XIX*, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the disease process or condition.

For the treatment of conditions and diseases relating to immunosuppression or neuronal damage, a compound of the invention may be administered orally, topically, parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intramuscular, and intrasternal injection or infusion techniques.

Dosage levels of the compounds of the present invention are of the order from about 0.01 mg to about 50 mg per kilogram of body weight per day, preferably from

about 0.1 mg to about 10 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated conditions (from about 0.7 mg to about 3.5 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the present invention may be administered on an intermittent basis, i.e., at semi-weekly, weekly, semi-monthly, or monthly intervals.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 g of active agent compounded with an appropriate and convenient amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds of the invention can be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001% to 10% by weight, and most preferably from about 0.005% to 0.8% by weight. The compounds and compositions of the invention are useful in treating disease conditions using doses and administration schedules as described for tacrolimus in U.S. Patent Nos. 5,542,436; 5,365,948; 5,348,966; and 5,196,437, incorporated herein by reference. The compounds of the invention can be used as single therapeutic agents or in combination with other therapeutic agents. Drugs that can be usefully combined with compounds of the invention include one or more immunosuppressant agents such as rapamycin, cyclosporin A, FK-506, or one or more neurotrophic agents.

It will be understood, however, that the specific dosage level for any particular patient will depend on a variety of factors. These factors include the activity of the specific compound employed; the age, body weight, general health, sex, and diet of the subject; the time and route of administration and the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the present invention and shall not be construed as being a limitation on the scope of the invention or claims.

Example 1

Replacement of Methoxyl with Hydrogen or Methyl at C-13 of FK-520

The C-13 methoxyl group is introduced into FK-520 via an AT domain in extender module 8 of the PKS that is specific for hydroxymalonyl and by methylation of the hydroxyl group by an S-adenosyl methionine (SAM) dependent methyltransferase. Metabolism of FK-506 and FK-520 primarily involves oxidation at the C-13 position into an inactive derivative that is further degraded by host P450 and other enzymes. The present invention provides compounds related in structure to FK-506 and FK-520 that do not contain the C-13 methoxy group and exhibit greater stability and a longer half-life *in vivo*. These compounds are useful medicaments due to their immunosuppressive and neurotrophic activities, and the invention provides the compounds in purified form and as pharmaceutical compositions.

The present invention also provides the novel PKS enzymes that produce these novel compounds as well as the expression vectors and host cells that produce the novel PKS enzymes. The novel PKS enzymes include, among others, those that contain an AT domain specific for either malonyl CoA or methylmalonyl CoA in module 8 of the FK-506 and FK-520 PKS. This example describes the construction of recombinant DNA compounds that encode the novel FK-520 PKS enzymes and the transformation of host cells with those recombinant DNA compounds to produce the novel PKS enzymes and the polyketides produced thereby.

To construct an expression cassette for performing module 8 AT domain replacements in the FK-520 PKS, a 4.6 kb *Sph*I fragment from the FK-520 gene cluster was cloned into plasmid pLitmus 38 (a cloning vector available from New England Biolabs). The 4.6 kb *Sph*I fragment, which encodes the ACP domain of module 7 followed by module 8 through the KR domain, was isolated from an agarose gel after digesting the cosmid pKOS65-C31 with *Sph* I. The clone having the insert oriented so

the single *Sac*I site was nearest to the *Spe*I end of the polylinker was identified and designated as plasmid pKOS60-21-67. To generate appropriate cloning sites, two linkers were ligated sequentially as follows. First, a linker was ligated between the *Spe*I and *Sac*I sites to introduce a *Bgl*II site at the 5' end of the cassette, to eliminate interfering polylinker sites, and to reduce the total insert size to 4.5 kb (the limit of the phage KC515). The ligation reactions contained 5 picomolar unphosphorylated linker DNA and 0.1 picomolar vector DNA, i.e., a 50-fold molar excess of linker to vector. The linker had the following sequence:

5'-CTAGTGGGCAGATCTGGCAGCT-3'
3'-ACCCGTCTAGACCG-5'

The resulting plasmid was designated pKOS60-27-1.

Next, a linker of the following sequence was ligated between the unique *Sph*I and *Afl*II sites of plasmid pKOS60-27-1 to introduce an *Nsi*I site at the 3' end of the module 8 cassette. The linker employed was:

5'-GGGATGCATGGC-3'
3'-GTACCCCTACGTACCGAATT-5'

The resulting plasmid was designated pKOS60-29-55.

To allow in-frame insertions of alternative AT domains, sites were engineered at the 5' end (*Avr* II or *Nhe* I) and 3' end (*Xho* I) of the AT domain using the polymerase chain reaction (PCR) as follows. Plasmid pKOS60-29-55 was used as a template for the PCR and sequence 5' to the AT domain was amplified with the primers SpeBgl-fwd and either Avr-rev or Nhe-rev:

SpeBgl-fwd 5'-CGACTCACTAGTGGGCAGATCTGG-3'

Avr-rev 5'-CACGCCTAGGCCGGTCGGTCTCGGGCCAC-3'

Nhe-rev 5'-GCGGCTAGCTGCTCGCCCATCGCGGGATGC-3'

The PCR included, in a 50 μl reaction, 5 μl of 10x *Pfu* polymerase buffer (Stratagene), 5 μl 10x z-dNTP mixture (2 mM dATP, 2 mM dCTP, 2 mM dTTP, 1 mM dGTP, 1 mM 7-deaza-GTP), 5 μl DMSO, 2 μl of each primer (10 μM), 1 μl of template DNA (0.1 μg/μl), and 1 μl of cloned *Pfu* polymerase (Stratagene). The PCR conditions were 95°C for 2 min., 25 cycles at 95°C for 30 sec., 60°C for 30 sec., and 72°C for 4

min., followed by 4 min. at 72°C and a hold at 0°C. The amplified DNA products and the Litmus vectors were cut with the appropriate restriction enzymes (*Bgl*II and *Avr*II or *Spe*I and *Nhe*I), and cloned into either pLitmus 28 or pLitmus38 (New England Biolabs), respectively, to generate the constructs designated pKOS60-37-4 and pKOS60-37-2, respectively.

Plasmid pKOS60-29-55 was again used as a template for PCR to amplify sequence 3' to the AT domain using the primers BsrXho-fwd and NsiAfl-rev:

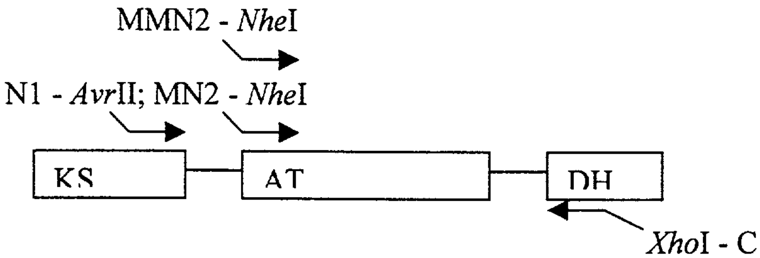
BsrXho-fwd 5'-GATGTACAGCTCGAGTCGGCACGCCCGGCCGCATC-3'

NsiAfl-rev 5'-CGACTCACTTAAGCCATGCATCC-3'

PCR conditions were as described above. The PCR fragment was cut with *Bsr*GI and *Afl*II, gel isolated, and ligated into pKOS60-37-4 cut with *Asp*718 and *Afl*II and inserted into pKOS60-37-2 cut with *Bsr*GI and *Afl*II, to give the plasmids pKOS60-39-1 and pKOS60-39-13, respectively. These two plasmids can be digested with *Avr*II and *Xho*I or *Nhe*I and *Xho*I, respectively, to insert heterologous AT domains specific for malonyl, methylmalonyl, ethylmalonyl, or other extender units.

Malonyl and methylmalonyl-specific AT domains were cloned from the rapamycin cluster using PCR amplification with a pair of primers that introduce an *Avr*II or *Nhe*I site at the 5' end and an *Xho*I site at the 3' end. The PCR conditions were as given above and the primer sequences were as follows:

RATN1 5'-ATCCTAGGCGGGCRGGYGTGTCGTCCTTCGG-3'
(3' end of Rap KS sequence and universal for malonyl and methylmalonyl CoA),
RATMN2 5'-ATGCTAGCCGCCGCGTTCCCCGTCTTCGCGCG-3'
(Rap AT shorter version 5'- sequence and specific for malonyl CoA),
RATMMN2 5'-ATGCTAGCGGATTCGTCGGTGGTGTTCGCCGA-3'
(Rap AT shorter version 5'- sequence and specific for methylmalonyl CoA), and
RATC 5'-ATCTCGAGCCAGTASCGCTGGTGYTGGAAGG-3'
(Rap DH 5'- sequence and universal for malonyl and methylmalonyl CoA).



Any Rap Module

Because of the high sequence similarity in each module of the rapamycin cluster, each primer was expected to prime any of the AT domains. PCR products representing ATs specific for malonyl or methylmalonyl extenders were identified by sequencing individual cloned PCR products. Sequencing also confirmed that the chosen clones contained no cloning artifacts. Examples of hybrid modules with the rapamycin AT12 and AT13 domains are shown in a separate figure.

The *Avr*II-*Xho*I restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 12 of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below. The AT of rap module 12 is specific for incorporation of malonyl units.

```
AGATCTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50
  I  W  Q  L  A  E  A  L  L  T  L  V  R  E  S  T
GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGGCGGC 100
 A  A  V  L  G  H  V  G  G  E  D  I  P  A  T  A  A
GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150
  F  K  D  L  G  I  D  S  L  T  A  V  Q  L  R  N
CCCTCACCGAGGCGACCGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200
A  L  T  E  A  T  G  V  R  L  N  A  T  A  V  F  D
TTCCCGACCCCGCACGTGCTCGCCGGGAAGCTCGGCGACGAACTGACCGG 250
 F  P  T  P  H  V  L  A  G  K  L  G  D  E  L  T  G
CACCCGCGCGCCCCGTCGTGCCCCGGACCGCGGCCACGGCCGGTGCGCACG 300
  T  R  A  P  V  V  P  R  T  A  A  T  A  G  A  H
ACGAGCCGCTGGCGATCGTGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350
D  E  P  L  A  I  V  G  M  A  C  R  L  P  G  G  V
GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400
 A  S  P  E  E  L  W  H  L  V  A  S  G  T  D  A  I
CACGGAGTTCCCGACGGACCGCGGCTGGGACGTCGACGCGATCTACGACC 450
  T  E  F  P  T  D  R  G  W  D  V  D  A  I  Y  D
CGGACCCCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500
P  D  P  D  A  I  G  K  T  F  V  R  H  G  G  F  L
ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550
 T  G  A  T  G  F  D  A  A  F  F  G  I  S  P  R  E
GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600
  A  L  A  M  D  P  Q  Q  R  V  L  L  E  T  S  W
AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGGCAGCGAC 650
```

```
 E  A  F  E  S  A  G  I  T  P  D  S  T  R  G  S  D
ACCGGCGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700
  T  G  V  F  V  G  A  F  S  Y  G  Y  G  T  G  A  D
CACCGACGGCTTCGGCGCGACCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750
   T  D  G  F  G  A  T  G  S  Q  T  S  V  L  S  G
GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800
 R  L  S  Y  F  Y  G  L  E  G  P  A  V  T  V  D  T
GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG 850
  A  C  S  S  S  L  V  A  L  H  Q  A  G  Q  S  L  R
CTCCGGCGAATGCTCGCTCGCCCTGGTCGGCGGCGTCACGGTGATGGCGT 900
   S  G  E  C  S  L  A  L  V  G  G  V  T  V  M  A
CTCCCGGCGGCTTCGTGGAGTTCTCCCGGCAGCGCGGCCTCGCGCCGGAC 950
 S  P  G  G  F  V  E  F  S  R  Q  R  G  L  A  P  D
GGCCGGGCGAAGGCGTTCGGCGCGGGTGCGGACGGCACGAGCTTCGCCGA 1000
  G  R  A  K  A  F  G  A  G  A  D  G  T  S  F  A  E
GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCGAACGCAACG 1050
   G  A  G  V  L  I  V  E  R  L  S  D  A  E  R  N
GTCACACCGTCCTGGCGGTCGTCCGTGGTTCGGCGGTCAACCAGGATGGT 1100
 G  H  T  V  L  A  V  V  R  G  S  A  V  N  Q  D  G
GCCTCCAACGGGCTGTCGGCGCCGAACGGGCCGTCGCAGGAGCGGGTGAT 1150
  A  S  N  G  L  S  A  P  N  G  P  S  Q  E  R  V  I
CCGGCAGGCCCTGGCCAACGCCGGGCTCACCCCGGCGGACGTGGACGCCG 1200
   R  Q  A  L  A  N  A  G  L  T  P  A  D  V  D  A
TCGAGGCCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG 1250
 V  E  A  H  G  T  G  T  R  L  G  D  P  I  E  A  Q
GCGGTACTGGCCACCTACGGACAGGAGCGCGCCACCCCCCTGCTGCTGGG 1300
  A  V  L  A  T  Y  G  Q  E  R  A  T  P  L  L  L  G
CTCGCTGAAGTCCAACATCGGCCACGCCCAGGCCGCGTCCGGCGTCGCCG 1350
   S  L  K  S  N  I  G  H  A  Q  A  A  S  G  V  A
GCATCATCAAGATGGTGCAGGCCCTCCGGCACGGGGAGCTGCCGCCGACG 1400
 G  I  I  K  M  V  Q  A  L  R  H  G  E  L  P  P  T
CTGCACGCCGACGAGCCGTCGCCGCACGTCGACTGGACGGCCGGCGCCGT 1450
  L  H  A  D  E  P  S  P  H  V  D  W  T  A  G  A  V
CGAACTGCTGACGTCGGCCCGGCCGTGGCCCGAGACCGACCGGCCTAGGC 1500
   E  L  L  T  S  A  R  P  W  P  E  T  D  R  P  R
GGGCAGGCGTGTCGTCCTTCGGGATCAGTGGCACCAACGCCCACGTCATC 1550
 R  A  G  V  S  S  F  G  I  S  G  T  N  A  H  V  I
CTGGAAAGCGCACCCCCCACTCAGCCTGCGGACAACGCGGTGATCGAGCG 1600
  L  E  S  A  P  P  T  Q  P  A  D  N  A  V  I  E  R
GGCACCGGAGTGGGTGCCGTTGGTGATTTCGGCCAGGACCCAGTCGGCTT 1650
   A  P  E  W  V  P  L  V  I  S  A  R  T  Q  S  A
TGACTGAGCACGAGGGCCGGTTGCGTGCGTATCTGGCGGCGTCGCCCGGG 1700
 L  T  E  H  E  G  R  L  R  A  Y  L  A  A  S  P  G
GTGGATATGCGGGCTGTGGCATCGACGCTGGCGATGACACGGTCGGTGTT 1750
  V  D  M  R  A  V  A  S  T  L  A  M  T  R  S  V  F
CGAGCACCGTGCCGTGCTGCTGGGAGATGACACCGTCACCGGCACCGCTG 1800
   E  H  R  A  V  L  L  G  D  D  T  V  T  G  T  A
TGTCTGACCCTCGGGCGGTGTTCGTCTTCCCGGGACAGGGGTCGCAGCGT 1850
 V  S  D  P  R  A  V  F  V  F  P  G  Q  G  S  Q  R
GCTGGCATGGGTGAGGAACTGGCCGCCGCGTTCCCCGTCTTCGCGCGGAT 1900
  A  G  M  G  E  E  L  A  A  A  F  P  V  F  A  R  I
CCATCAGCAGGTGTGGGACCTGCTCGATGTGCCCGATCTGGAGGTGAACG 1950
   H  Q  Q  V  W  D  L  L  D  V  P  D  L  E  V  N
AGACCGGTTACGCCCAGCCGGCCCTGTTCGCAATGCAGGTGGCTCTGTTC 2000
```

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E  T  G  Y  A  Q  P  A  L  F  A  M  Q  V  A  L  F
GGGCTGCTGGAATCGTGGGGTGTACGACCGGACGCGGTGATCGGCCATTC 2050
 G  L  L  E  S  W  G  V  R  P  D  A  V  I  G  H  S
GGTGGGTGAGCTTGCGGCTGCGTATGTGTCCGGGGTGTGGTCGTTGGAGG 2100
 V  G  E  L  A  A  Y  V  S  G  V  W  S  L  E
ATGCCTGCACTTTGGTGTCGGCGCGGGCTCGTCTGATGCAGGCTCTGCCC 2150
D  A  C  T  L  V  S  A  R  A  R  L  M  Q  A  L  P
GCGGGTGGGGTGATGGTCGCTGTCCCGGTCTCGGAGGATGAGGCCCGGGC 2200
 A  G  G  V  M  V  A  V  P  V  S  E  D  E  A  R  A
CGTGCTGGGTGAGGGTGTGGAGATCGCCGCGGTCAACGGCCCGTCGTCGG 2250
  V  L  G  E  G  V  E  I  A  A  V  N  G  P  S  S
TGGTTCTCTCCGGTGATGAGGCCGCCGTGCTGCAGGCCGCGGAGGGGCTG 2300
V  V  L  S  G  D  E  A  A  V  L  Q  A  A  E  G  L
GGGAAGTGGACGCGGCTGGCGACCAGCCACGCGTTCCATTCCGCCCGTAT 2350
 G  K  W  T  R  L  A  T  S  H  A  F  H  S  A  R  M
GGAACCCATGCTGGAGGAGTTCCGGGCGGTCGCCGAAGGCCTGACCTACC 2400
  E  P  M  L  E  E  F  R  A  V  A  E  G  L  T  Y
GGACGCCGCAGGTCTCCATGGCCGTTGGTGATCAGGTGACCACCGCTGAG 2450
R  T  P  Q  V  S  M  A  V  G  D  Q  V  T  T  A  E
TACTGGGTGCGGCAGGTCCGGGACACGGTCCGGTTCGGCGAGCAGGTGGC 2500
 Y  W  V  R  Q  V  R  D  T  V  R  F  G  E  Q  V  A
CTCGTACGAGGACGCCGTGTTCGTCGAGCTGGGTGCCGACCGGTCACTGG 2550
  S  Y  E  D  A  V  F  V  E  L  G  A  D  R  S  L
CCCGCCTGGTCGACGGTGTCGCGATGCTGCACGGCGACCACGAAATCCAG 2600
A  R  L  V  D  G  V  A  M  L  H  G  D  H  E  I  Q
GCCGCGATCGGCGCCCTGGCCCACCTGTATGTCAACGGCGTCACGGTCGA 2650
 A  A  I  G  A  L  A  H  L  Y  V  N  G  V  T  V  D
CTGGCCCGCGCTCCTGGGCGATGCTCCGGCAACACGGGTGCTGGACCTTC 2700
  W  P  A  L  L  G  D  A  P  A  T  R  V  L  D  L
CGACATACGCCTTCCAGCACCAGCGCTACTGGCTCGAGTCGGCACGCCCG 2750
P  T  Y  A  F  Q  H  Q  R  Y  W  L  E  S  A  R  P
GCCGCATCCGACGCGGGCCACCCCGTGCTGGGCTCCGGTATCGCCCTCGC 2800
 A  A  S  D  A  G  H  P  V  L  G  S  G  I  A  L  A
CGGGTCGCCGGGCCGGGTGTTCACGGGTTCCGTGCCGACCGGTGCGGACC 2850
  G  S  P  G  R  V  F  T  G  S  V  P  T  G  A  D
GCGCGGTGTTCGTCGCCGAGCTGGCGCTGGCCGCCGCGGACGCGGTCGAC 2900
R  A  V  F  V  A  E  L  A  L  A  A  A  D  A  V  D
TGCGCCACGGTCGAGCGGCTCGACATCGCCTCCGTGCCCGGCCGGCCGGG 2950
 C  A  T  V  E  R  L  D  I  A  S  V  P  G  R  P  G
CCATGGCCGGACGACCGTACAGACCTGGGTCGACGAGCCGGCGGACGACG 3000
  H  G  R  T  T  V  Q  T  W  V  D  E  P  A  D  D
GCCGGCGCCGGTTCACCGTGCACACCCGCACCGGCGACGCCCCGTGGACG 3050
G  R  R  R  F  T  V  H  T  R  T  G  D  A  P  W  T
CTGCACGCCGAGGGGGTGCTGCGCCCCCATGGCACGGCCCTGCCCGATGC 3100
 L  H  A  E  G  V  L  R  P  H  G  T  A  L  P  D  A
GGCCGACGCCGAGTGGCCCCCACCGGGCGCGGTGCCCGCGGACGGGCTGC 3150
  A  D  A  E  W  P  P  P  G  A  V  P  A  D  G  L
CGGGTGTGTGGCGCCGGGGGGACCAGGTCTTCGCCGAGGCCGAGGTGGAC 3200
P  G  V  W  R  R  G  D  Q  V  F  A  E  A  E  V  D
GGACCGGACGGTTTCGTGGTGCACCCCGACCTGCTCGACGCGGTCTTCTC 3250
 G  P  D  G  F  V  V  H  P  D  L  L  D  A  V  F  S
CGCGGTCGGCGACGGAAGCCGCCAGCCGGCCGGATGGCGCGACCTGACGG 3300
  A  V  G  D  G  S  R  Q  P  A  G  W  R  D  L  T
TGCACGCGTCGGACGCCACCGTACTGCGCGCCTGCCTCACCCGGCGCACC 3350
```

```
V   H   A   S   D   A   T   V   L   R   A   C   L   T   R   R   T
GACGGAGCCATGGGATTCGCCGCCTTCGACGGCGCCGGCCTGCCGGTACT 3400
 D   G   A   M   G   F   A   A   F   D   G   A   G   L   P   V   L
CACCGCGGAGGCGGTGACGCTGCGGGAGGTGGCGTCACCGTCCGGCTCCG 3450
  T   A   E   A   V   T   L   R   E   V   A   S   P   S   G   S
AGGAGTCGGACGGCCTGCACCGGTTGGAGTGGCTCGCGGTCGCCGAGGCG 3500
E   E   S   D   G   L   H   R   L   E   W   L   A   V   A   E   A
GTCTACGACGGTGACCTGCCCGAGGGACATGTCCTGATCACCGCCGCCCA 3550
 V   Y   D   G   D   L   P   E   G   H   V   L   I   T   A   A   H
CCCCGACGACCCCGAGGACATACCCACCCGCGCCCACACCCGCGCCACCC 3600
  P   D   D   P   E   D   I   P   T   R   A   H   T   R   A   T
GCGTCCTGACCGCCCTGCAACACCACCTCACCACCACCGACCACACCCTC 3650
R   V   L   T   A   L   Q   H   H   L   T   T   T   D   H   T   L
ATCGTCCACACCACCACCGACCCCGCCGGCGCCACCGTCACCGGCCTCAC 3700
 I   V   H   T   T   T   D   P   A   G   A   T   V   T   G   L   T
CCGCACCGCCCAGAACGAACACCCCCACCGCATCCGCCTCATCGAAACCG 3750
  R   T   A   Q   N   E   H   P   H   R   I   R   L   I   E   T
ACCACCCCCACACCCCCCTCCCCCTGGCCCAACTCGCCACCCTCGACCAC 3800
D   H   P   H   T   P   L   P   L   A   Q   L   A   T   L   D   H
CCCCACCTCCGCCTCACCCACCACACCCTCCACCACCCCCACCTCACCCC 3850
 P   H   L   R   L   T   H   H   T   L   H   H   P   H   L   T   P
CCTCCACACCACCACCCCACCCACCACCACCCCCCTCAACCCCGAACACG 3900
  L   H   T   T   T   P   P   T   T   T   P   L   N   P   E   H
CCATCATCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCCGC 3950
A   I   I   I   T   G   G   S   G   T   L   A   G   I   L   A   R
CACCTGAACCACCCCCACACCTACCTCCTCTCCCGCACCCCACCCCCCGA 4000
 H   L   N   H   P   H   T   Y   L   L   S   R   T   P   P   P   D
CGCCACCCCCGGCACCCACCTCCCCTGCGACGTCGGCGACCCCCACCAAC 4050
  A   T   P   G   T   H   L   P   C   D   V   G   D   P   H   Q
TCGCCACCACCCTCACCCACATCCCCCAACCCCTCACCGCCATCTTCCAC 4100
L   A   T   T   L   T   H   I   P   Q   P   L   T   A   I   F   H
ACCGCCGCCACCCTCGACGACGGCATCCTCCACGCCCTCACCCCCGACCG 4150
 T   A   A   T   L   D   D   G   I   L   H   A   L   T   P   D   R
CCTCACCACCGTCCTCCACCCCAAAGCCAACGCCGCCTGGCACCTGCACC 4200
  L   T   T   V   L   H   P   K   A   N   A   A   W   H   L   H
ACCTCACCCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCAGCGCC 4250
H   L   T   Q   N   Q   P   L   T   H   F   V   L   Y   S   S   A
GCCGCCGTCCTCGGCAGCCCCGGACAAGGAAACTACGCCGCCGCCAACGC 4300
 A   A   V   L   G   S   P   G   Q   G   N   Y   A   A   A   N   A
CTTCCTCGACGCCCTCGCCACCCACCGCCACACCCTCGGCCAACCCGCCA 4350
  F   L   D   A   L   A   T   H   R   H   T   L   G   Q   P   A
CCTCCATCGCCTGGGGCATGTGGCACACCACCAGCACCCTCACCGGACAA 4400
T   S   I   A   W   G   M   W   H   T   T   S   T   L   T   G   Q
CTCGACGACGCCGACCGGGACCGCATCCGCCGCGGCGGTTTCCTCCCGAT 4450
 L   D   D   A   D   R   D   R   I   R   R   G   G   F   L   P   I
CACGGACGACGAGGGCATGGGGATGCAT
  T   D   D   E   G
```

The *Avr*II-*Xho*I restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 13 (specific for

methylmalonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

```
AGATCTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50
 Q  L  A  E  A  L  L  T  L  V  R  E  S  T
GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGGCGGC 100
 A  A  V  L  G  H  V  G  G  E  D  I  P  A  T  A  A
GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150
  F  K  D  L  G  I  D  S  L  T  A  V  Q  L  R  N
CCCTCACCGAGGCGACCGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200
A  L  T  E  A  T  G  V  R  L  N  A  T  A  V  F  D
TTCCCGACCCCGCACGTGCTCGCCGGGAAGCTCGGCGACGAACTGACCGG 250
 F  P  T  P  H  V  L  A  G  K  L  G  D  E  L  T  G
CACCCGCGCGCCCGTCGTGCCCCGGACCGCGGCCACGGCCGGTGCGCACG 300
  T  R  A  P  V  V  P  R  T  A  A  T  A  G  A  H
ACGAGCCGCTGGCGATCGTGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350
D  E  P  L  A  I  V  G  M  A  C  R  L  P  G  G  V
GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400
 A  S  P  E  E  L  W  H  L  V  A  S  G  T  D  A  I
CACGGAGTTCCCGACGGACCGCGGCTGGGACGTCGACGCGATCTACGACC 450
  T  E  F  P  T  D  R  G  W  D  V  D  A  I  Y  D
CGGACCCCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500
P  D  P  D  A  I  G  K  T  F  V  R  H  G  G  F  L
ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550
 T  G  A  T  G  F  D  A  A  F  F  G  I  S  P  R  E
GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600
  A  L  A  M  D  P  Q  Q  R  V  L  L  E  T  S  W
AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGGCAGCGAC 650
E  A  F  E  S  A  G  I  T  P  D  S  T  R  G  S  D
ACCGGCGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700
 T  G  V  F  V  G  A  F  S  Y  G  Y  G  T  G  A  D
CACCGACGGCTTCGGCGCGACCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750
  T  D  G  F  G  A  T  G  S  Q  T  S  V  L  S  G
GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800
R  L  S  Y  F  Y  G  L  E  G  P  A  V  T  V  D  T
GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG 850
 A  C  S  S  S  L  V  A  L  H  Q  A  G  Q  S  L  R
CTCCGGCGAATGCTCGCTCGCCCTGGTCGGCGGCGTCACGGTGATGGCGT 900
  S  G  E  C  S  L  A  L  V  G  G  V  T  V  M  A
CTCCCGGCGGCTTCGTGGAGTTCTCCCGGCAGCGCGGCCTCGCGCCGGAC 950
S  P  G  G  F  V  E  F  S  R  Q  R  G  L  A  P  D
GGCCGGGCGAAGGCGTTCGGCGCGGGTGCGGACGGCACGAGCTTCGCCGA 1000
 G  R  A  K  A  F  G  A  G  A  D  G  T  S  F  A  E
GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCGAACGCAACG 1050
  G  A  G  V  L  I  V  E  R  L  S  D  A  E  R  N
GTCACACCGTCCTGGCGGTCGTCCGTGGTTCGGCGGTCAACCAGGATGGT 1100
G  H  T  V  L  A  V  V  R  G  S  A  V  N  Q  D  G
GCCTCCAACGGGCTGTCGGCGCCGAACGGGCCGTCGCAGGAGCGGGTGAT 1150
 A  S  N  G  L  S  A  P  N  G  P  S  Q  E  R  V  I
CCGGCAGGCCCTGGCCAACGCCGGGCTCACCCCGGCGGACGTGGACGCCG 1200
  R  Q  A  L  A  N  A  G  L  T  P  A  D  V  D  A
TCGAGGCCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG 1250
V  E  A  H  G  T  G  T  R  L  G  D  P  I  E  A  Q
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GCGGTACTGGCCACCTACGGACAGGAGCGCGCCACCCCCCTGCTGCTGGG 1300
 A  V  L  A  T  Y  G  Q  E  R  A  T  P  L  L  L  G
CTCGCTGAAGTCCAACATCGGCCACGCCCAGGCCGCGTCCGGCGTCGCCG 1350
  S  L  K  S  N  I  G  H  A  Q  A  A  S  G  V  A
GCATCATCAAGATGGTGCAGGCCCTCCGGCACGGGGAGCTGCCGCCGACG 1400
G  I  I  K  M  V  Q  A  L  R  H  G  E  L  P  P  T
CTGCACGCCGACGAGCCGTCGCCGCACGTCGACTGGACGGCCGGCGCCGT 1450
 L  H  A  D  E  P  S  P  H  V  D  W  T  A  G  A  V
CGAACTGCTGACGTCGGCCCGGCCGTGGCCCGAGACCGACCGGCCTAGGC 1500
  E  L  L  T  S  A  R  P  W  P  E  T  D  R  P  R
GGGCGGGCGTGTCGTCCTTCGGAGTCAGCGGCACCAACGCCCACGTCATC 1550
R  A  G  V  S  S  F  G  V  S  G  T  N  A  H  V  I
CTGGAGAGCGCACCCCCCGCTCAGCCCGCGGAGGAGGCGCAGCCTGTTGA 1600
 L  E  S  A  P  P  A  Q  P  A  E  E  A  Q  P  V  E
GACGCCGGTGGTGGCCTCGGATGTGCTGCCGCTGGTGATATCGGCCAAGA 1650
  T  P  V  V  A  S  D  V  L  P  L  V  I  S  A  K
CCCAGCCCGCCCTGACCGAACACGAAGACCGGCTGCGCGCCTACCTGGCG 1700
T  Q  P  A  L  T  E  H  E  D  R  L  R  A  Y  L  A
GCGTCGCCCGGGGCGGATATACGGGCTGTGGCATCGACGCTGGCGGTGAC 1750
 A  S  P  G  A  D  I  R  A  V  A  S  T  L  A  V  T
ACGGTCGGTGTTCGAGCACCGCGCCGTACTCCTTGGAGATGACACCGTCA 1800
  R  S  V  F  E  H  R  A  V  L  L  G  D  D  T  V
CCGGCACCGCGGTGACCGACCCCAGGATCGTGTTTGTCTTTCCCGGGCAG 1850
T  G  T  A  V  T  D  P  R  I  V  F  V  F  P  G  Q
GGGTGGCAGTGGCTGGGGATGGGCAGTGCACTGCGCGATTCGTCGGTGGT 1900
 G  W  Q  W  L  G  M  G  S  A  L  R  D  S  S  V  V
GTTCGCCGAGCGGATGGCCGAGTGTGCGGCGGCGTTGCGCGAGTTCGTGG 1950
  F  A  E  R  M  A  E  C  A  A  A  L  R  E  F  V
ACTGGGATCTGTTCACGGTTCTGGATGATCCGGCGGTGGTGGACCGGGTT 2000
D  W  D  L  F  T  V  L  D  D  P  A  V  V  D  R  V
GATGTGGTCCAGCCCGCTTCCTGGGCGATGATGGTTTCCCTGGCCGCGGT 2050
 D  V  V  Q  P  A  S  W  A  M  M  V  S  L  A  A  V
GTGGCAGGCGGCCGGTGTGCGGCCGGATGCGGTGATCGGCCATTCGCAGG 2100
  W  Q  A  A  G  V  R  P  D  A  V  I  G  H  S  Q
GTGAGATCGCCGCAGCTTGTGTGGCGGGTGCGGTGTCACTACGCGATGCC 2150
G  E  I  A  A  A  C  V  A  G  A  V  S  L  R  D  A
GCCCGGATCGTGACCTTGCGCAGCCAGGCGATCGCCCGGGGCCTGGCGGG 2200
 A  R  I  V  T  L  R  S  Q  A  I  A  R  G  L  A  G
CCGGGGCGCGATGGCATCCGTCGCCCTGCCCGCGCAGGATGTCGAGCTGG 2250
  R  G  A  M  A  S  V  A  L  P  A  Q  D  V  E  L
TCGACGGGGCCTGGATCGCCGCCCACAACGGGCCCGCCTCCACCGTGATC 2300
V  D  G  A  W  I  A  A  H  N  G  P  A  S  T  V  I
GCGGGCACCCCGGAAGCGGTCGACCATGTCCTCACCGCTCATGAGGCACA 2350
 A  G  T  P  E  A  V  D  H  V  L  T  A  H  E  A  Q
AGGGGTGCGGGTGCGGCGGATCACCGTCGACTATGCCTCGCACACCCCGC 2400
  G  V  R  V  R  R  I  T  V  D  Y  A  S  H  T  P
ACGTCGAGCTGATCCGCGACGAACTACTCGACATCACTAGCGACAGCAGC 2450
H  V  E  L  I  R  D  E  L  L  D  I  T  S  D  S  S
TCGCAGACCCCGCTCGTGCCGTGGCTGTCGACCGTGGACGGCACCTGGGT 2500
 S  Q  T  P  L  V  P  W  L  S  T  V  D  G  T  W  V
CGACAGCCCGCTGGACGGGGAGTACTGGTACCGGAACCTGCGTGAACCGG 2550
  D  S  P  L  D  G  E  Y  W  Y  R  N  L  R  E  P
TCGGTTTCCACCCCGCCGTCAGCCAGTTGCAGGCCCAGGGCGACACCGTG 2600
V  G  F  H  P  A  V  S  Q  L  Q  A  Q  G  D  T  V
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TTCGTCGAGGTCAGCGCCAGCCCGGTGTTGTTGCAGGCGATGGACGACGA 2650
 F  V  E  V  S  A  S  P  V  L  L  Q  A  M  D  D  D
TGTCGTCACGGTTGCCACGCTGCGTCGTGACGACGGCGACGCCACCCGGA 2700
  V  V  T  V  A  T  L  R  R  D  D  G  D  A  T  R
TGCTCACCGCCCTGGCACAGGCCTATGTCCACGGCGTCACCGTCGACTGG 2750
M  L  T  A  L  A  Q  A  Y  V  H  G  V  T  V  D  W
CCCGCCATCCTCGGCACCACCACAACCCGGGTACTGGACCTTCCGACCTA 2800
 P  A  I  L  G  T  T  T  T  R  V  L  D  L  P  T  Y
CGCCTTCCAACACCAGCGGTACTGGCTCGAGTCGGCACGCCCGGCCGCAT 2850
  A  F  Q  H  Q  R  Y  W  L  E  S  A  R  P  A  A
CCGACGCGGGCCACCCCGTGCTGGGCTCCGGTATCGCCCTCGCCGGGTCG 2900
S  D  A  G  H  P  V  L  G  S  G  I  A  L  A  G  S
CCGGGCCGGGTGTTCACGGGTTCCGTGCCGACCGGTGCGGACCGCGCGGT 2950
 P  G  R  V  F  T  G  S  V  P  T  G  A  D  R  A  V
GTTCGTCGCCGAGCTGGCGCTGGCCGCCGCGGACGCGGTCGACTGCGCCA 3000
  F  V  A  E  L  A  L  A  A  A  D  A  V  D  C  A
CGGTCGAGCGGCTCGACATCGCCTCCGTGCCCGGCCGGCCGGGCCATGGC 3050
T  V  E  R  L  D  I  A  S  V  P  G  R  P  G  H  G
CGGACGACCGTACAGACCTGGGTCGACGAGCCGGCGGACGACGGCCGGCG 3100
 R  T  T  V  Q  T  W  V  D  E  P  A  D  D  G  R  R
CCGGTTCACCGTGCACACCCGCACCGGCGACGCCCCGTGGACGCTGCACG 3150
  R  F  T  V  H  T  R  T  G  D  A  P  W  T  L  H
CCGAGGGGGTGCTGCGCCCCCATGGCACGGCCCTGCCCGATGCGGCCGAC 3200
A  E  G  V  L  R  P  H  G  T  A  L  P  D  A  A  D
GCCGAGTGGCCCCCACCGGGCGCGGTGCCCGCGGACGGGCTGCCGGGTGT 3250
 A  E  W  P  P  P  G  A  V  P  A  D  G  L  P  G  V
GTGGCGCCGGGGGGACCAGGTCTTCGCCGAGGCCGAGGTGGACGGACCGG 3300
  W  R  R  G  D  Q  V  F  A  E  A  E  V  D  G  P
ACGGTTTCGTGGTGCACCCCGACCTGCTCGACGCGGTCTTCTCCGCGGTC 3350
D  G  F  V  V  H  P  D  L  L  D  A  V  F  S  A  V
GGCGACGGAAGCCGCCAGCCGGCCGGATGGCGCGACCTGACGGTGCACGC 3400
 G  D  G  S  R  Q  P  A  G  W  R  D  L  T  V  H  A
GTCGGACGCCACCGTACTGCGCGCCTGCCTCACCCGGCGCACCGACGGAG 3450
  S  D  A  T  V  L  R  A  C  L  T  R  R  T  D  G
CCATGGGATTCGCCGCCTTCGACGGCGCCGGCCTGCCGGTACTCACCGCG 3500
A  M  G  F  A  A  F  D  G  A  G  L  P  V  L  T  A
GAGGCGGTGACGCTGCGGGAGGTGGCGTCACCGTCCGGCTCCGAGGAGTC 3550
 E  A  V  T  L  R  E  V  A  S  P  S  G  S  E  E  S
GGACGGCCTGCACCGGTTGGAGTGGCTCGCGGTCGCCGAGGCGGTCTACG 3600
  D  G  L  H  R  L  E  W  L  A  V  A  E  A  V  Y
ACGGTGACCTGCCCGAGGGACATGTCCTGATCACCGCCGCCCACCCCGAC 3650
D  G  D  L  P  E  G  H  V  L  I  T  A  A  H  P  D
GACCCCGAGGACATACCCACCCGCGCCCACACCCGCGCCACCCGCGTCCT 3700
 D  P  E  D  I  P  T  R  A  H  T  R  A  T  R  V  L
GACCGCCCTGCAACACCACCTCACCACCACCGACCACACCCTCATCGTCC 3750
  T  A  L  Q  H  H  L  T  T  T  D  H  T  L  I  V
ACACCACCACCGACCCCGCCGGCGCCACCGTCACCGGCCTCACCCGCACC 3800
H  T  T  T  D  P  A  G  A  T  V  T  G  L  T  R  T
GCCCAGAACGAACACCCCCACCGCATCCGCCTCATCGAAACCGACCACCC 3850
 A  Q  N  E  H  P  H  R  I  R  L  I  E  T  D  H  P
CCACACCCCCCTCCCCCTGGCCCAACTCGCCACCCTCGACCACCCCCACC 3900
  H  T  P  L  P  L  A  Q  L  A  T  L  D  H  P  H
TCCGCCTCACCCACCACACCCTCCACCACCCCCACCTCACCCCCCTCCAC 3950
L  R  L  T  H  H  T  L  H  H  P  H  L  T  P  L  H
```

```
ACCACCACCCCACCCACCACCACCCCCCTCAACCCCGAACACGCCATCAT 4000
 T  T  T  P  P  T  T  T  P  L  N  P  E  H  A  I  I
CATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCCGCCACCTGA 4050
  I  T  G  G  S  G  T  L  A  G  I  L  A  R  H  L
ACCACCCCCACACCTACCTCCTCTCCCGCACCCCACCCCCCGACGCCACC 4100
N  H  P  H  T  Y  L  L  S  R  T  P  P  P  D  A  T
CCCGGCACCCACCTCCCCTGCGACGTCGGCGACCCCCACCAACTCGCCAC 4150
 P  G  T  H  L  P  C  D  V  G  D  P  H  Q  L  A  T
CACCCTCACCCACATCCCCCAACCCCTCACCGCCATCTTCCACACCGCCG 4200
  T  L  T  H  I  P  Q  P  L  T  A  I  F  H  T  A
CCACCCTCGACGACGGCATCCTCCACGCCCTCACCCCCGACCGCCTCACC 4250
A  T  L  D  D  G  I  L  H  A  L  T  P  D  R  L  T
ACCGTCCTCCACCCCAAAGCCAACGCCGCCTGGCACCTGCACCACCTCAC 4300
 T  V  L  H  P  K  A  N  A  A  W  H  L  H  H  L  T
CCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCAGCGCCGCCGCCG 4350
  Q  N  Q  P  L  T  H  F  V  L  Y  S  S  A  A  A
TCCTCGGCAGCCCCGGACAAGGAAACTACGCCGCCGCCAACGCCTTCCTC 4400
V  L  G  S  P  G  Q  G  N  Y  A  A  A  N  A  F  L
GACGCCCTCGCCACCCACCGCCACACCCTCGGCCAACCCGCCACCTCCAT 4450
 D  A  L  A  T  H  R  H  T  L  G  Q  P  A  T  S  I
CGCCTGGGGCATGTGGCACACCACCAGCACCCTCACCGGACAACTCGACG 4500
  A  W  G  M  W  H  T  T  S  T  L  T  G  Q  L  D
ACGCCGACCGGGACCGCATCCGCCGCGGCGGTTTCCTCCCGATCACGGAC 4550
D  A  D  R  D  R  I  R  R  G  G  F  L  P  I  T  D
GACGAGGGCATGGGGATGCAT
 D  E  G
```

The *Nhe*II-*Xho*I restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 12 (specific for malonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

```
AGATCTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50
 Q  L  A  E  A  L  L  T  L  V  R  E  S  T
GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGGCGGC 100
 A  A  V  L  G  H  V  G  G  E  D  I  P  A  T  A  A
GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150
  F  K  D  L  G  I  D  S  L  T  A  V  Q  L  R  N
CCCTCACCGAGGCGACCGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200
A  L  T  E  A  T  G  V  R  L  N  A  T  A  V  F  D
TTCCCGACCCCGCACGTGCTCGCCGGGAAGCTCGGCGACGAACTGACCGG 250
 F  P  T  P  H  V  L  A  G  K  L  G  D  E  L  T  G
CACCCGCGCGCCCGTCGTGCCCCGGACCGCGGCCACGGCCGGTGCGCACG 300
  T  R  A  P  V  V  P  R  T  A  A  T  A  G  A  H
ACGAGCCGCTGGCGATCGTGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350
D  E  P  L  A  I  V  G  M  A  C  R  L  P  G  G  V
GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400
 A  S  P  E  E  L  W  H  L  V  A  S  G  T  D  A  I
CACGGAGTTCCCGACGGACCGCGGCTGGGACGTCGACGCGATCTACGACC 450
  T  E  F  P  T  D  R  G  W  D  V  D  A  I  Y  D
CGGACCCCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500
```

```
P  D  P  D  A  I  G  K  T  F  V  R  H  G  G  F  L
ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550
 T  G  A  T  G  F  D  A  A  F  F  G  I  S  P  R  E
GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600
  A  L  A  M  D  P  Q  Q  R  V  L  L  E  T  S  W
AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGGCAGCGAC 650
E  A  F  E  S  A  G  I  T  P  D  S  T  R  G  S  D
ACCGGCGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700
 T  G  V  F  V  G  A  F  S  Y  G  Y  G  T  G  A  D
CACCGACGGCTTCGGCGCGACCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750
  T  D  G  F  G  A  T  G  S  Q  T  S  V  L  S  G
GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800
R  L  S  Y  F  Y  G  L  E  G  P  A  V  T  V  D  T
GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG 850
 A  C  S  S  S  L  V  A  L  H  Q  A  G  Q  S  L  R
CTCCGGCGAATGCTCGCTCGCCCTGGTCGGCGGCGTCACGGTGATGGCGT 900
  S  G  E  C  S  L  A  L  V  G  G  V  T  V  M  A
CTCCCGGCGGCTTCGTGGAGTTCTCCCGGCAGCGCGGCCTCGCGCCGGAC 950
S  P  G  G  F  V  E  F  S  R  Q  R  G  L  A  P  D
GGCCGGGCGAAGGCGTTCGGCGCGGGTGCGGACGGCACGAGCTTCGCCGA 1000
 G  R  A  K  A  F  G  A  G  A  D  G  T  S  F  A  E
GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCGAACGCAACG 1050
  G  A  G  V  L  I  V  E  R  L  S  D  A  E  R  N
GTCACACCGTCCTGGCGGTCGTCCGTGGTTCGGCGGTCAACCAGGATGGT 1100
G  H  T  V  L  A  V  V  R  G  S  A  V  N  Q  D  G
GCCTCCAACGGGCTGTCGGCGCCGAACGGGCCGTCGCAGGAGCGGGTGAT 1150
 A  S  N  G  L  S  A  P  N  G  P  S  Q  E  R  V  I
CCGGCAGGCCCTGGCCAACGCCGGGCTCACCCCGGCGGACGTGGACGCCG 1200
  R  Q  A  L  A  N  A  G  L  T  P  A  D  V  D  A
TCGAGGCCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG 1250
V  E  A  H  G  T  G  T  R  L  G  D  P  I  E  A  Q
GCGGTACTGGCCACCTACGGACAGGAGCGCGCCACCCCCCTGCTGCTGGG 1300
 A  V  L  A  T  Y  G  Q  E  R  A  T  P  L  L  L  G
CTCGCTGAAGTCCAACATCGGCCACGCCCAGGCCGCGTCCGGCGTCGCCG 1350
  S  L  K  S  N  I  G  H  A  Q  A  A  S  G  V  A
GCATCATCAAGATGGTGCAGGCCCTCCGGCACGGGGAGCTGCCGCCGACG 1400
G  I  I  K  M  V  Q  A  L  R  H  G  E  L  P  P  T
CTGCACGCCGACGAGCCGTCGCCGCACGTCGACTGGACGGCCGGCGCCGT 1450
 L  H  A  D  E  P  S  P  H  V  D  W  T  A  G  A  V
CGAACTGCTGACGTCGGCCCGGCCGTGGCCCGAGACCGACCGGCCACGGC 1500
  E  L  L  T  S  A  R  P  W  P  E  T  D  R  P  R
GTGCCGCCGTCTCCTCGTTCGGGGTGAGCGGCACCAACGCCCACGTCATC 1550
R  A  A  V  S  S  F  G  V  S  G  T  N  A  H  V  I
CTGGAGGCCGGACCGGTAACGGAGACGCCCGCGGCATCGCCTTCCGGTGA 1600
 L  E  A  G  P  V  T  E  T  P  A  A  S  P  S  G  D
CCTTCCCCTGCTGGTGTCGGCACGCTCACCGGAAGCGCTCGACGAGCAGA 1650
  L  P  L  L  V  S  A  R  S  P  E  A  L  D  E  Q
TCCGCCGACTGCGCGCCTACCTGGACACCACCCCGGACGTCGACCGGGTG 1700
I  R  R  L  R  A  Y  L  D  T  T  P  D  V  D  R  V
GCCGTGGCACAGACGCTGGCCCGGCGCACACACTTCGCCCACCGCGCCGT 1750
 A  V  A  Q  T  L  A  R  R  T  H  F  A  H  R  A  V
GCTGCTCGGTGACACCGTCATCACCACACCCCCCGCGGACCGGCCCGACG 1800
  L  L  G  D  T  V  I  T  T  P  P  A  D  R  P  D
AACTCGTCTTCGTCTACTCCGGCCAGGGCACCCAGCATCCCGCGATGGGC 1850
```

E L V F V Y S G Q G T Q H P A M G
GAGCAGCTAGCCGCCGCGTTCCCCGTCTTCGCGCGGATCCATCAGCAGGT 1900
E Q L A A A F P V F A R I H Q Q V
GTGGGACCTGCTCGATGTGCCCGATCTGGAGGTGAACGAGACCGGTTACG 1950
W D L L D V P D L E V N E T G Y
CCCAGCCGGCCCTGTTCGCAATGCAGGTGGCTCTGTTCGGGCTGCTGGAA 2000
A Q P A L F A M Q V A L F G L L E
TCGTGGGGTGTACGACCGGACGCGGTGATCGGCCATTCGGTGGGTGAGCT 2050
S W G V R P D A V I G H S V G E L
TGCGGCTGCGTATGTGTCCGGGGTGTGGTCGTTGGAGGATGCCTGCACTT 2100
A A A Y V S G V W S L E D A C T
TGGTGTCGGCGCGGGCTCGTCTGATGCAGGCTCTGCCCGCGGGTGGGGTG 2150
L V S A R A R L M Q A L P A G G V
ATGGTCGCTGTCCCGGTCTCGGAGGATGAGGCCCGGGCCGTGCTGGGTGA 2200
M V A V P V S E D E A R A V L G E
GGGTGTGGAGATCGCCGCGGTCAACGGCCCGTCGTCGGTGGTTCTCTCCG 2250
G V E I A A V N G P S S V V L S
GTGATGAGGCCGCCGTGCTGCAGGCCGCGGAGGGGCTGGGGAAGTGGACG 2300
G D E A A V L Q A A E G L G K W T
CGGCTGGCGACCAGCCACGCGTTCCATTCCGCCCGTATGGAACCCATGCT 2350
R L A T S H A F H S A R M E P M L
GGAGGAGTTCCGGGCGGTCGCCGAAGGCCTGACCTACCGGACGCCGCAGG 2400
E E F R A V A E G L T Y R T P Q
TCTCCATGGCCGTTGGTGATCAGGTGACCACCGCTGAGTACTGGGTGCGG 2450
V S M A V G D Q V T T A E Y W V R
CAGGTCCGGGACACGGTCCGGTTCGGCGAGCAGGTGGCCTCGTACGAGGA 2500
Q V R D T V R F G E Q V A S Y E D
CGCCGTGTTCGTCGAGCTGGGTGCCGACCGGTCACTGGCCCGCCTGGTCG 2550
A V F V E L G A D R S L A R L V
ACGGTGTCGCGATGCTGCACGGCGACCACGAAATCCAGGCCGCGATCGGC 2600
D G V A M L H G D H E I Q A A I G
GCCCTGGCCCACCTGTATGTCAACGGCGTCACGGTCGACTGGCCCGCGCT 2650
A L A H L Y V N G V T V D W P A L
CCTGGGCGATGCTCCGGCAACACGGGTGCTGGACCTTCCGACATACGCCT 2700
L G D A P A T R V L D L P T Y A
TCCAGCACCAGCGCTACTGGCTCGAGTCGGCACGCCCGGCCGCATCCGAC 2750
F Q H Q R Y W L E S A R P A A S D
GCGGGCCACCCCGTGCTGGGCTCCGGTATCGCCCTCGCCGGGTCGCCGGG 2800
A G H P V L G S G I A L A G S P G
CCGGGTGTTCACGGGTTCCGTGCCGACCGGTGCGGACCGCGCGGTGTTCG 2850
R V F T G S V P T G A D R A V F
TCGCCGAGCTGGCGCTGGCCGCCGCGGACGCGGTCGACTGCGCCACGGTC 2900
V A E L A L A A A D A V D C A T V
GAGCGGCTCGACATCGCCTCCGTGCCCGGCCGGCCGGGCCATGGCCGGAC 2950
E R L D I A S V P G R P G H G R T
GACCGTACAGACCTGGGTCGACGAGCCGGCGGACGACGGCCGGCGCCGGT 3000
T V Q T W V D E P A D D G R R R
TCACCGTGCACACCCGCACCGGCGACGCCCCGTGGACGCTGCACGCCGAG 3050
F T V H T R T G D A P W T L H A E
GGGGTGCTGCGCCCCCATGGCACGGCCCTGCCCGATGCGGCCGACGCCGA 3100
G V L R P H G T A L P D A A D A E
GTGGCCCCCACCGGGCGCGGTGCCCGCGGACGGGCTGCCGGGTGTGTGGC 3150
W P P P G A V P A D G L P G V W
GCCGGGGGGACCAGGTCTTCGCCGAGGCCGAGGTGGACGGACCGGACGGT 3200

```
 R  R  G  D  Q  V  F  A  E  A  E  V  D  G  P  D  G
TTCGTGGTGCACCCCGACCTGCTCGACGCGGTCTTCTCCGCGGTCGGCGA 3250
  F  V  V  H  P  D  L  L  D  A  V  F  S  A  V  G  D
CGGAAGCCGCCAGCCGGCCGGATGGCGCGACCTGACGGTGCACGCGTCGG 3300
   G  S  R  Q  P  A  G  W  R  D  L  T  V  H  A  S
ACGCCACCGTACTGCGCGCCTGCCTCACCCGGCGCACCGACGGAGCCATG 3350
 D  A  T  V  L  R  A  C  L  T  R  R  T  D  G  A  M
GGATTCGCCGCCTTCGACGGCGCCGGCCTGCCGGTACTCACCGCGGAGGC 3400
  G  F  A  A  F  D  G  A  G  L  P  V  L  T  A  E  A
GGTGACGCTGCGGGAGGTGGCGTCACCGTCCGGCTCCGAGGAGTCGGACG 3450
   V  T  L  R  E  V  A  S  P  S  G  S  E  E  S  D
GCCTGCACCGGTTGGAGTGGCTCGCGGTCGCCGAGGCGGTCTACGACGGT 3500
 G  L  H  R  L  E  W  L  A  V  A  E  A  V  Y  D  G
GACCTGCCCGAGGGACATGTCCTGATCACCGCCGCCCACCCCGACGACCC 3550
  D  L  P  E  G  H  V  L  I  T  A  A  H  P  D  D  P
CGAGGACATACCCACCCGCGCCCACACCCGCGCCACCCGCGTCCTGACCG 3600
   E  D  I  P  T  R  A  H  T  R  A  T  R  V  L  T
CCCTGCAACACCACCTCACCACCACCGACCACACCCTCATCGTCCACACC 3650
 A  L  Q  H  H  L  T  T  T  D  H  T  L  I  V  H  T
ACCACCGACCCCGCCGGCGCCACCGTCACCGGCCTCACCCGCACCGCCCA 3700
  T  T  D  P  A  G  A  T  V  T  G  L  T  R  T  A  Q
GAACGAACACCCCCACCGCATCCGCCTCATCGAAACCGACCACCCCCACA 3750
   N  E  H  P  H  R  I  R  L  I  E  T  D  H  P  H
CCCCCCTCCCCCTGGCCCAACTCGCCACCCTCGACCACCCCCACCTCCGC 3800
 T  P  L  P  L  A  Q  L  A  T  L  D  H  P  H  L  R
CTCACCCACCACACCCTCCACCACCCCCACCTCACCCCCCTCCACACCAC 3850
  L  T  H  H  T  L  H  H  P  H  L  T  P  L  H  T  T
CACCCCACCCACCACCACCCCCCTCAACCCCGAACACGCCATCATCATCA 3900
   T  P  P  T  T  T  P  L  N  P  E  H  A  I  I  I
CCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCCGCCACCTGAACCAC 3950
 T  G  G  S  G  T  L  A  G  I  L  A  R  H  L  N  H
CCCCACACCTACCTCCTCTCCCGCACCCCACCCCCCGACGCCACCCCCGG 4000
  P  H  T  Y  L  L  S  R  T  P  P  P  D  A  T  P  G
CACCCACCTCCCCTGCGACGTCGGCGACCCCCACCAACTCGCCACCACCC 4050
   T  H  L  P  C  D  V  G  D  P  H  Q  L  A  T  T
TCACCCACATCCCCCAACCCCTCACCGCCATCTTCCACACCGCCGCCACC 4100
 L  T  H  I  P  Q  P  L  T  A  I  F  H  T  A  A  T
CTCGACGACGGCATCCTCCACGCCCTCACCCCCGACCGCCTCACCACCGT 4150
  L  D  D  G  I  L  H  A  L  T  P  D  R  L  T  T  V
CCTCCACCCCAAAGCCAACGCCGCCTGGCACCTGCACCACCTCACCCAAA 4200
   L  H  P  K  A  N  A  A  W  H  L  H  H  L  T  Q
ACCAACCCCTCACCCACTTCGTCCTCTACTCCAGCGCCGCCGCCGTCCTC 4250
 N  Q  P  L  T  H  F  V  L  Y  S  S  A  A  A  V  L
GGCAGCCCCGGACAAGGAAACTACGCCGCCGCCAACGCCTTCCTCGACGC 4300
  G  S  P  G  Q  G  N  Y  A  A  A  N  A  F  L  D  A
CCTCGCCACCCACCGCCACACCCTCGGCCAACCCGCCACCTCCATCGCCT 4350
   L  A  T  H  R  H  T  L  G  Q  P  A  T  S  I  A
GGGGCATGTGGCACACCACCAGCACCCTCACCGGACAACTCGACGACGCC 4400
 W  G  M  W  H  T  T  S  T  L  T  G  Q  L  D  D  A
GACCGGGACCGCATCCGCCGCGGCGGTTTCCTCCCGATCACGGACGACGA 4450
  D  R  D  R  I  R  R  G  G  F  L  P  I  T  D  D  E
GGGCATGGGGATGCAT
   G
```

The *Nhe*II-*Xho*I restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 13 (specific for methylmalonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

```
AGATCTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50
 Q  L  A  E  A  L  L  T  L  V  R  E  S  T
GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGGCGGC 100
 A  A  V  L  G  H  V  G  G  E  D  I  P  A  T  A  A
GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150
  F  K  D  L  G  I  D  S  L  T  A  V  Q  L  R  N
CCCTCACCGAGGCGACCGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200
A  L  T  E  A  T  G  V  R  L  N  A  T  A  V  F  D
TTCCCGACCCCGCACGTGCTCGCCGGGAAGCTCGGCGACGAACTGACCGG 250
 F  P  T  P  H  V  L  A  G  K  L  G  D  E  L  T  G
CACCCGCGCGCCCGTCGTGCCCCGGACCGCGGCCACGGCCGGTGCGCACG 300
  T  R  A  P  V  V  P  R  T  A  A  T  A  G  A  H
ACGAGCCGCTGGCGATCGTGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350
D  E  P  L  A  I  V  G  M  A  C  R  L  P  G  G  V
GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400
 A  S  P  E  E  L  W  H  L  V  A  S  G  T  D  A  I
CACGGAGTTCCCGACGGACCGCGGCTGGGACGTCGACGCGATCTACGACC 450
  T  E  F  P  T  D  R  G  W  D  V  D  A  I  Y  D
CGGACCCCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500
P  D  P  D  A  I  G  K  T  F  V  R  H  G  G  F  L
ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550
 T  G  A  T  G  F  D  A  A  F  F  G  I  S  P  R  E
GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600
  A  L  A  M  D  P  Q  Q  R  V  L  L  E  T  S  W
AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGGCAGCGAC 650
E  A  F  E  S  A  G  I  T  P  D  S  T  R  G  S  D
ACCGGCGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700
 T  G  V  F  V  G  A  F  S  Y  G  Y  G  T  G  A  D
CACCGACGGCTTCGGCGCGACCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750
  T  D  G  F  G  A  T  G  S  Q  T  S  V  L  S  G
GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800
R  L  S  Y  F  Y  G  L  E  G  P  A  V  T  V  D  T
GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG 850
 A  C  S  S  S  L  V  A  L  H  Q  A  G  Q  S  L  R
CTCCGGCGAATGCTCGCTCGCCCTGGTCGGCGGCGTCACGGTGATGGCGT 900
  S  G  E  C  S  L  A  L  V  G  G  V  T  V  M  A
CTCCCGGCGGCTTCGTGGAGTTCTCCCGGCAGCGCGGCCTCGCGCCGGAC 950
S  P  G  G  F  V  E  F  S  R  Q  R  G  L  A  P  D
GGCCGGGCGAAGGCGTTCGGCGCGGGTGCGGACGGCACGAGCTTCGCCGA 1000
 G  R  A  K  A  F  G  A  G  A  D  G  T  S  F  A  E
GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCGAACGCAACG 1050
  G  A  G  V  L  I  V  E  R  L  S  D  A  E  R  N
GTCACACCGTCCTGGCGGTCGTCCGTGGTTCGGCGGTCAACCAGGATGGT 1100
G  H  T  V  L  A  V  V  R  G  S  A  V  N  Q  D  G
GCCTCCAACGGGCTGTCGGCGCCGAACGGGCCGTCGCAGGAGCGGGTGAT 1150
 A  S  N  G  L  S  A  P  N  G  P  S  Q  E  R  V  I
```

```
CCGGCAGGCCCTGGCCAACGCCGGGCTCACCCCGGCGGACGTGGACGCCG 1200
  R  Q  A  L  A  N  A  G  L  T  P  A  D  V  D  A
TCGAGGCCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG 1250
V  E  A  H  G  T  G  T  R  L  G  D  P  I  E  A  Q
GCGGTACTGGCCACCTACGGACAGGAGCGCGCCACCCCCCTGCTGCTGGG 1300
 A  V  L  A  T  Y  G  Q  E  R  A  T  P  L  L  L  G
CTCGCTGAAGTCCAACATCGGCCACGCCCAGGCCGCGTCCGGCGTCGCCG 1350
  S  L  K  S  N  I  G  H  A  Q  A  A  S  G  V  A
GCATCATCAAGATGGTGCAGGCCCTCCGGCACGGGGAGCTGCCGCCGACG 1400
G  I  I  K  M  V  Q  A  L  R  H  G  E  L  P  P  T
CTGCACGCCGACGAGCCGTCGCCGCACGTCGACTGGACGGCCGGCGCCGT 1450
 L  H  A  D  E  P  S  P  H  V  D  W  T  A  G  A  V
CGAACTGCTGACGTCGGCCCGGCCGTGGCCCGAGACCGACCGGCCACGGC 1500
  E  L  L  T  S  A  R  P  W  P  E  T  D  R  P  R
GTGCCGCCGTCTCCTCGTTCGGGGTGAGCGGCACCAACGCCCACGTCATC 1550
R  A  A  V  S  S  F  G  V  S  G  T  N  A  H  V  I
CTGGAGGCCGGACCGGTAACGGAGACGCCCGCGGCATCGCCTTCCGGTGA 1600
 L  E  A  G  P  V  T  E  T  P  A  A  S  P  S  G  D
CCTTCCCCTGCTGGTGTCGGCACGCTCACCGGAAGCGCTCGACGAGCAGA 1650
  L  P  L  L  V  S  A  R  S  P  E  A  L  D  E  Q
TCCGCCGACTGCGCGCCTACCTGGACACCACCCCGGACGTCGACCGGGTG 1700
I  R  R  L  R  A  Y  L  D  T  T  P  D  V  D  R  V
GCCGTGGCACAGACGCTGGCCCGGCGCACACACTTCGCCCACCGCGCCGT 1750
 A  V  A  Q  T  L  A  R  R  T  H  F  A  H  R  A  V
GCTGCTCGGTGACACCGTCATCACCACACCCCCCGCGGACCGGCCCGACG 1800
  L  L  G  D  T  V  I  T  T  P  P  A  D  R  P  D
AACTCGTCTTCGTCTACTCCGGCCAGGGCACCCAGCATCCCGCGATGGGC 1850
E  L  V  F  V  Y  S  G  Q  G  T  Q  H  P  A  M  G
GAGCAGCTAGCCGATTCGTCGGTGGTGTTCGCCGAGCGGATGGCCGAGTG 1900
 E  Q  L  A  D  S  S  V  V  F  A  E  R  M  A  E  C
TGCGGCGGCGTTGCGCGAGTTCGTGGACTGGGATCTGTTCACGGTTCTGG 1950
  A  A  A  L  R  E  F  V  D  W  D  L  F  T  V  L
ATGATCCGGCGGTGGTGGACCGGGTTGATGTGGTCCAGCCCGCTTCCTGG 2000
D  D  P  A  V  V  D  R  V  D  V  V  Q  P  A  S  W
GCGATGATGGTTTCCCTGGCCGCGGTGTGGCAGGCGGCCGGTGTGCGGCC 2050
 A  M  M  V  S  L  A  A  V  W  Q  A  A  G  V  R  P
GGATGCGGTGATCGGCCATTCGCAGGGTGAGATCGCCGCAGCTTGTGTGG 2100
  D  A  V  I  G  H  S  Q  G  E  I  A  A  A  C  V
CGGGTGCGGTGTCACTACGCGATGCCGCCCGGATCGTGACCTTGCGCAGC 2150
A  G  A  V  S  L  R  D  A  A  R  I  V  T  L  R  S
CAGGCGATCGCCCGGGGCCTGGCGGGCCGGGGCGCGATGGCATCCGTCGC 2200
 Q  A  I  A  R  G  L  A  G  R  G  A  M  A  S  V  A
CCTGCCCGCGCAGGATGTCGAGCTGGTCGACGGGGCCTGGATCGCCGCCC 2250
  L  P  A  Q  D  V  E  L  V  D  G  A  W  I  A  A
ACAACGGGCCCGCCTCCACCGTGATCGCGGGCACCCCGGAAGCGGTCGAC 2300
H  N  G  P  A  S  T  V  I  A  G  T  P  E  A  V  D
CATGTCCTCACCGCTCATGAGGCACAAGGGGTGCGGGTGCGGCGGATCAC 2350
 H  V  L  T  A  H  E  A  Q  G  V  R  V  R  R  I  T
CGTCGACTATGCCTCGCACACCCCGCACGTCGAGCTGATCCGCGACGAAC 2400
  V  D  Y  A  S  H  T  P  H  V  E  L  I  R  D  E
TACTCGACATCACTAGCGACAGCAGCTCGCAGACCCCGCTCGTGCCGTGG 2450
L  L  D  I  T  S  D  S  S  S  Q  T  P  L  V  P  W
CTGTCGACCGTGGACGGCACCTGGGTCGACAGCCCGCTGGACGGGGAGTA 2500
 L  S  T  V  D  G  T  W  V  D  S  P  L  D  G  E  Y
```

```
CTGGTACCGGAACCTGCGTGAACCGGTCGGTTTCCACCCCGCCGTCAGCC 2550
 W  Y  R  N  L  R  E  P  V  G  F  H  P  A  V  S
AGTTGCAGGCCCAGGGCGACACCGTGTTCGTCGAGGTCAGCGCCAGCCCG 2600
Q  L  Q  A  Q  G  D  T  V  F  V  E  V  S  A  S  P
GTGTTGTTGCAGGCGATGGACGACGATGTCGTCACGGTTGCCACGCTGCG 2650
 V  L  L  Q  A  M  D  D  D  V  V  T  V  A  T  L  R
TCGTGACGACGGCGACGCCACCCGGATGCTCACCGCCCTGGCACAGGCCT 2700
 R  D  D  G  D  A  T  R  M  L  T  A  L  A  Q  A
ATGTCCACGGCGTCACCGTCGACTGGCCCGCCATCCTCGGCACCACCACA 2750
Y  V  H  G  V  T  V  D  W  P  A  I  L  G  T  T  T
ACCCGGGTACTGGACCTTCCGACCTACGCCTTCCAACACCAGCGGTACTG 2800
 T  R  V  L  D  L  P  T  Y  A  F  Q  H  Q  R  Y  W
GCTCGAGTCGGCACGCCCGGCCGCATCCGACGCGGGCCACCCCGTGCTGG 2850
 L  E  S  A  R  P  A  A  S  D  A  G  H  P  V  L
GCTCCGGTATCGCCCTCGCCGGGTCGCCGGGCCGGGTGTTCACGGGTTCC 2900
G  S  G  I  A  L  A  G  S  P  G  R  V  F  T  G  S
GTGCCGACCGGTGCGGACCGCGCGGTGTTCGTCGCCGAGCTGGCGCTGGC 2950
 V  P  T  G  A  D  R  A  V  F  V  A  E  L  A  L  A
CGCCGCGGACGCGGTCGACTGCGCCACGGTCGAGCGGCTCGACATCGCCT 3000
 A  A  D  A  V  D  C  A  T  V  E  R  L  D  I  A
CCGTGCCCGGCCGGCCGGGCCATGGCCGGACGACCGTACAGACCTGGGTC 3050
S  V  P  G  R  P  G  H  G  R  T  T  V  Q  T  W  V
GACGAGCCGGCGGACGACGGCCGGCGCCGGTTCACCGTGCACACCCGCAC 3100
 D  E  P  A  D  D  G  R  R  R  F  T  V  H  T  R  T
CGGCGACGCCCCGTGGACGCTGCACGCCGAGGGGGTGCTGCGCCCCCATG 3150
 G  D  A  P  W  T  L  H  A  E  G  V  L  R  P  H
GCACGGCCCTGCCCGATGCGGCCGACGCCGAGTGGCCCCCACCGGGCGCG 3200
G  T  A  L  P  D  A  A  D  A  E  W  P  P  P  G  A
GTGCCCGCGGACGGGCTGCCGGGTGTGTGGCGCCGGGGGGACCAGGTCTT 3250
 V  P  A  D  G  L  P  G  V  W  R  R  G  D  Q  V  F
CGCCGAGGCCGAGGTGGACGGACCGGACGGTTTCGTGGTGCACCCCGACC 3300
 A  E  A  E  V  D  G  P  D  G  F  V  V  H  P  D
TGCTCGACGCGGTCTTCTCCGCGGTCGGCGACGGAAGCCGCCAGCCGGCC 3350
L  L  D  A  V  F  S  A  V  G  D  G  S  R  Q  P  A
GGATGGCGCGACCTGACGGTGCACGCGTCGGACGCCACCGTACTGCGCGC 3400
 G  W  R  D  L  T  V  H  A  S  D  A  T  V  L  R  A
CTGCCTCACCCGGCGCACCGACGGAGCCATGGGATTCGCCGCCTTCGACG 3450
 C  L  T  R  R  T  D  G  A  M  G  F  A  A  F  D
GCGCCGGCCTGCCGGTACTCACCGCGGAGGCGGTGACGCTGCGGGAGGTG 3500
G  A  G  L  P  V  L  T  A  E  A  V  T  L  R  E  V
GCGTCACCGTCCGGCTCCGAGGAGTCGGACGGCCTGCACCGGTTGGAGTG 3550
 A  S  P  S  G  S  E  E  S  D  G  L  H  R  L  E  W
GCTCGCGGTCGCCGAGGCGGTCTACGACGGTGACCTGCCCGAGGGACATG 3600
 L  A  V  A  E  A  V  Y  D  G  D  L  P  E  G  H
TCCTGATCACCGCCGCCCACCCCGACGACCCCGAGGACATACCCACCCGC 3650
V  L  I  T  A  A  H  P  D  D  P  E  D  I  P  T  R
GCCCACACCCGCGCCACCCGCGTCCTGACCGCCCTGCAACACCACCTCAC 3700
 A  H  T  R  A  T  R  V  L  T  A  L  Q  H  H  L  T
CACCACCGACCACACCCTCATCGTCCACACCACCACCGACCCCGCCGGCG 3750
 T  T  D  H  T  L  I  V  H  T  T  T  D  P  A  G
CCACCGTCACCGGCCTCACCCGCACCGCCCAGAACGAACACCCCCACCGC 3800
A  T  V  T  G  L  T  R  T  A  Q  N  E  H  P  H  R
ATCCGCCTCATCGAAACCGACCACCCCCACACCCCCCTCCCCCTGGCCCA 3850
 I  R  L  I  E  T  D  H  P  H  T  P  L  P  L  A  Q
```

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ACTCGCCACCCTCGACCACCCCCACCTCCGCCTCACCCACCACACCCTCC 3900
 L  A  T  L  D  H  P  H  L  R  L  T  H  H  T  L
ACCACCCCCACCTCACCCCCCTCCACACCACCACCCCACCCACCACCACC 3950
H  H  P  H  L  T  P  L  H  T  T  T  P  P  T  T  T
CCCCTCAACCCCGAACACGCCATCATCATCACCGGCGGCTCCGGCACCCT 4000
 P  L  N  P  E  H  A  I  I  I  T  G  G  S  G  T  L
CGCCGGCATCCTCGCCCGCCACCTGAACCACCCCCACACCTACCTCCTCT 4050
 A  G  I  L  A  R  H  L  N  H  P  H  T  Y  L  L
CCCGCACCCCACCCCCCGACGCCACCCCCGGCACCCACCTCCCCTGCGAC 4100
S  R  T  P  P  P  D  A  T  P  G  T  H  L  P  C  D
GTCGGCGACCCCCACCAACTCGCCACCACCCTCACCCACATCCCCCAACC 4150
 V  G  D  P  H  Q  L  A  T  T  L  T  H  I  P  Q  P
CCTCACCGCCATCTTCCACACCGCCGCCACCCTCGACGACGGCATCCTCC 4200
 L  T  A  I  F  H  T  A  A  T  L  D  D  G  I  L
ACGCCCTCACCCCCGACCGCCTCACCACCGTCCTCCACCCCAAAGCCAAC 4250
H  A  L  T  P  D  R  L  T  T  V  L  H  P  K  A  N
GCCGCCTGGCACCTGCACCACCTCACCCAAAACCAACCCCTCACCCACTT 4300
 A  A  W  H  L  H  H  L  T  Q  N  Q  P  L  T  H  F
CGTCCTCTACTCCAGCGCCGCCGCCGTCCTCGGCAGCCCCGGACAAGGAA 4350
 V  L  Y  S  S  A  A  A  V  L  G  S  P  G  Q  G
ACTACGCCGCCGCCAACGCCTTCCTCGACGCCCTCGCCACCCACCGCCAC 4400
N  Y  A  A  A  N  A  F  L  D  A  L  A  T  H  R  H
ACCCTCGGCCAACCCGCCACCTCCATCGCCTGGGGCATGTGGCACACCAC 4450
 T  L  G  Q  P  A  T  S  I  A  W  G  M  W  H  T  T
CAGCACCCTCACCGGACAACTCGACGACGCCGACCGGGACCGCATCCGCC 4500
 S  T  L  T  G  Q  L  D  D  A  D  R  D  R  I  R
GCGGCGGTTTCCTCCCGATCACGGACGACGAGGGCATGGGGATGCAT
R  G  G  F  L  P  I  T  D  D  E  G
```

Phage KC515 DNA was prepared using the procedure described in Genetic Manipulation of *Streptomyces*, A Laboratory Manual, edited by D. Hopwood *et al.* A phage suspension prepared from 10 plates (100 mm) of confluent plaques of KC515 on *S. lividans* TK24 generally gave about 3 μg of phage DNA. The DNA was ligated to circularize at the cos site, subsequently digested with restriction enzymes *Bam*HI and *Pst*I, and dephosphorylated with SAP.

Each module 8 cassette described above was excised with restriction enzymes *Bgl*II and *Nsi*I and ligated into the compatible *Bam*HI and *Pst*I sites of KC515 phage DNA prepared as described above. The ligation mixture containing KC515 and various cassettes was transfected into protoplasts of *Streptomyces lividans* TK24 using the procedure described in Genetic Manipulation of *Streptomyces*, A Laboratory Manual edited by D. Hopwood *et al.* and overlaid with TK24 spores. After 16-24 hr, the plaques were restreaked on plates overlaid with TK24 spores. Single plaques were picked and resuspended in 200 μL of nutrient broth. Phage DNA was prepared by the boiling method

(Hopwood *et al.*, *supra*). The PCR with primers spanning the left and right boundaries of the recombinant phage was used to verify the correct phage had been isolated. In most cases, at least 80% of the plaques contained the expected insert. To confirm the presence of the resistance marker (thiostrepton), a spot test is used, as described in Lomovskaya *et al.* (1997), in which a plate with spots of phage is overlaid with mixture of spores of TK24 and phiC31 TK24 lysogen. After overnight incubation, the plate is overlaid with antibiotic in soft agar. A working stock is made of all phage containing desired constructs.

Streptomyces hygroscopicus ATCC 14891 (see US Patent No. 3,244,592, issued 5 Apr 1966, incorporated herein by reference) mycelia were infected with the recombinant phage by mixing the spores and phage (1×10^8 of each), and incubating on R2YE agar (Genetic Manipulation of *Streptomyces*, A Laboratory Manual, edited by D. Hopwood *et al.*) at 30°C for 10 days. Recombinant clones were selected and plated on minimal medium containing thiostrepton (50 μg/ml) to select for the thiostrepton resistance-conferring gene. Primary thiostrepton resistant clones were isolated and purified through a second round of single colony isolation, as necessary. To obtain thiostrepton-sensitive revertants that underwent a second recombination event to evict the phage genome, primary recombinants were propagated in liquid media for two to three days in the absence of thiostrepton and then spread on agar medium without thiostrepton to obtain spores. Spores were plated to obtain about 50 colonies per plate, and thiostrepton sensitive colonies were identified by replica plating onto thiostrepton containing agar medium. The PCR was used to determine which of the thiostrepton sensitive colonies reverted to the wild type (reversal of the initial integration event), and which contain the desired AT swap at module 8 in the ATCC 14891-derived cells. The PCR primers used amplified either the KS/AT junction or the AT/DH junction of the wild-type and the desired recombinant strains. Fermentation of the recombinant strains, followed by isolation of the metabolites and analysis by LCMS, and NMR is used to characterize the novel polyketide compounds.

Example 2

Replacement of Methoxyl with Hydrogen or Methyl at C-13 of FK-506

The present invention also provides the 13-desmethoxy derivatives of FK-506 and the novel PKS enzymes that produce them. A variety of *Streptomyces* strains that produce FK-506 are known in the art, including *S. tsukubaensis* No. 9993 (FERM BP-927), described in U.S. Patent No. 5,624,852, incorporated herein by reference; *S. hygroscopicus* subsp. *yakushimaensis* No. 7238, described in U.S. patent No. 4,894,366, incorporated herein by reference; *S.* sp. MA6858 (ATCC 55098), described in U.S. Patent Nos. 5,116,756, incorporated herein by reference; and *S.* sp. MA 6548, described in Motamedi *et al.*, 1998, "The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506," *Eur. J. Biochem. 256*: 528-534, and Motamedi *et al.*, 1997, "Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506," *Eur. J. Biochem. 244*: 74-80, each of which is incorporated herein by reference.

The complete sequence of the FK-506 gene cluster from *Streptomyces* sp. MA6548 is known, and the sequences of the corresponding gene clusters from other FK-506-producing organisms is highly homologous thereto. The novel FK-506 recombinant gene clusters of the present invention differ from the naturally occurring gene clusters in that the AT domain of module 8 of the naturally occurring PKSs is replaced by an AT domain specific for malonyl CoA or methylmalonyl CoA. These AT domain replacements are made at the DNA level, following the methodology described in Example 1.

The naturally occurring module 8 sequence for the MA6548 strain is shown below, followed by the illustrative hybrid module 8 sequences for the MA6548 strains.

```
GCATGCGGCTGTACGAGGCGGCACGGCGCACCGGAAGTCCCGTGGTGGTG 50
   M  R  L  Y  E  A  A  R  R  T  G  S  P  V  V  V
GCGGCCGCGCTCGACGACGCGCCGGACGTGCCGCTGCTGCGCGGGCTGCG 100
 A  A  A  L  D  D  A  P  D  V  P  L  L  R  G  L  R
GCGTACGACCGTCCGGCGTGCCGCCGTCCGGGAACGCTCTCTCGCCGACC 150
  R  T  T  V  R  R  A  A  V  R  E  R  S  L  A  D
GCTCGCCGTGCTGCCCGACGACGAGCGCGCCGACGCCTCCCTCGCGTTCG 200
R  S  P  C  C  P  T  T  S  A  P  T  P  P  S  R  S
TCCTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCGCCGAAGACAT 250
 S  W  N  S  T  A  T  V  L  G  H  L  G  A  E  D  I
```

```
CCCGGCGACGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300
 P  A  T  T  T  F  K  E  L  G  I  D  S  L  T  A
TCCAGCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350
V  Q  L  R  N  A  L  T  T  A  T  G  V  R  L  N  A
ACAGCGGTCTTCGACTTTCCGACGCCGCGCGCGCTCGCCGCGAGACTCGG 400
 T  A  V  F  D  F  P  T  P  R  A  L  A  A  R  L  G
CGACGAGCTGGCCGGTACCCGCGCGCCCGTCGCGGCCCGGACCGCGGCCA 450
  D  E  L  A  G  T  R  A  P  V  A  A  R  T  A  A
CCGCGGCCGCGCACGACGAACCGCTGGCGATCGTGGGCATGGCCTGCCGT 500
T  A  A  A  H  D  E  P  L  A  I  V  G  M  A  C  R
CTGCCGGGCGGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550
 L  P  G  G  V  A  S  P  Q  E  L  W  R  L  V  A  S
CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG 600
  G  T  D  A  I  T  E  F  P  A  D  R  G  W  D  V
ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650
D  A  L  Y  D  P  D  P  D  A  I  G  K  T  F  V  R
CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGGCGTTCTTCGG 700
 H  G  G  F  L  D  G  A  T  G  F  D  A  A  F  F  G
GATCAGCCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750
  I  S  P  R  E  A  L  A  M  D  P  Q  Q  R  V  L
TGGAGACGTCCTGGGAGGCGTTCGAAAGCGCGGGCATCACCCCGGACGCG 800
L  E  T  S  W  E  A  F  E  S  A  G  I  T  P  D  A
GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850
 A  R  G  S  D  T  G  V  F  I  G  A  F  S  Y  G  Y
CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900
  G  T  G  A  D  T  N  G  F  G  A  T  G  S  Q  T
GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
S  V  L  S  G  R  L  S  Y  F  Y  G  L  E  G  P  S
GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000
 V  T  V  D  T  A  C  S  S  S  L  V  A  L  H  Q  A
AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050
  G  Q  S  L  R  S  G  E  C  S  L  A  L  V  G  G
TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100
V  T  V  M  A  S  P  G  G  F  V  E  F  S  R  Q  R
GGGCTCGCGCCGGACGGGCGGGCGAAGGCGTTCGGCGCGGGCGCGGACGG 1150
 G  L  A  P  D  G  R  A  K  A  F  G  A  G  A  D  G
TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG 1200
  T  S  F  A  E  G  A  G  A  L  V  V  E  R  L  S
ACGCGGAGCGCCACGGCCACACCGTCCTCGCCCTCGTACGCGGCTCCGCG 1250
D  A  E  R  H  G  H  T  V  L  A  L  V  R  G  S  A
GCTAACTCCGACGGCGCGTCGAACGGTCTGTCGGCGCCGAACGGCCCCTC 1300
 A  N  S  D  G  A  S  N  G  L  S  A  P  N  G  P  S
CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350
  Q  E  R  V  I  H  Q  A  L  A  N  A  K  L  T  P
CCGATGTCGACGCGGTCGAGGCGCACGGCACCGGCACCCGCCTCGGCGAC 1400
A  D  V  D  A  V  E  A  H  G  T  G  T  R  L  G  D
CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGCGAC 1450
 P  I  E  A  Q  A  L  L  A  T  Y  G  Q  D  R  A  T
GCCCCTGCTGCTCGGCTCGCTGAAGTCGAACATCGGGCACGCCCAGGCCG 1500
  P  L  L  L  G  S  L  K  S  N  I  G  H  A  Q  A
CGTCAGGGGTCGCCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGGG 1550
A  S  G  V  A  G  I  I  K  M  V  Q  A  I  R  H  G
GAACTGCCGCCGACACTGCACGCGGACGAGCCGTCGCCGCACGTCGACTG 1600
 E  L  P  P  T  L  H  A  D  E  P  S  P  H  V  D  W
```

```
GACGGCCGGTGCCGTCGAGCTCCTGACGTCGGCCCGGCCGTGGCCGGGGA 1650
  T  A  G  A  V  E  L  L  T  S  A  R  P  W  P  G
CCGGTCGCCCGCGCCGCGCTGCCGTCTCGTCGTTCGGCGTGAGCGGCACG 1700
T  G  R  P  R  R  A  A  V  S  S  F  G  V  S  G  T
AACGCCCACATCATCCTTGAGGCAGGACCGGTCAAAACGGGACCGGTCGA 1750
 N  A  H  I  I  L  E  A  G  P  V  K  T  G  P  V  E
GGCAGGAGCGATCGAGGCAGGACCGGTCGAAGTAGGACCGGTCGAGGCTG 1800
  A  G  A  I  E  A  G  P  V  E  V  G  P  V  E  A
GACCGCTCCCCGCGGCGCCGCCGTCAGCACCGGGCGAAGACCTTCCGCTG 1850
G  P  L  P  A  A  P  P  S  A  P  G  E  D  L  P  L
CTCGTGTCGGCGCGTTCCCCGGAGGCACTCGACGAGCAGATCGGGCGCCT 1900
 L  V  S  A  R  S  P  E  A  L  D  E  Q  I  G  R  L
GCGCGCCTATCTCGACACCGGCCCGGGCGTCGACCGGGCGGCCGTGGCGC 1950
  R  A  Y  L  D  T  G  P  G  V  D  R  A  A  V  A
AGACACTGGCCCGGCGTACGCACTTCACCCACCGGGCCGTACTGCTCGGG 2000
Q  T  L  A  R  R  T  H  F  T  H  R  A  V  L  L  G
GACACCGTCATCGGCGCTCCCCCCGCGGACCAGGCCGACGAACTCGTCTT 2050
 D  T  V  I  G  A  P  P  A  D  Q  A  D  E  L  V  F
CGTCTACTCCGGTCAGGGCACCCAGCATCCCGCGATGGGCGAGCAACTCG 2100
  V  Y  S  G  Q  G  T  Q  H  P  A  M  G  E  Q  L
CGGCCGCGTTCCCCGTGTTCGCCGATGCCTGGCACGACGCGCTCCGACGG 2150
A  A  A  F  P  V  F  A  D  A  W  H  D  A  L  R  R
CTCGACGACCCCGACCCGCACGACCCCACACGGAGCCAGCACACGCTCTT 2200
 L  D  D  P  D  P  H  D  P  T  R  S  Q  H  T  L  F
CGCCCACCAGGCGGCGTTCACCGCCCTCCTGAGGTCCTGGGACATCACGC 2250
  A  H  Q  A  A  F  T  A  L  L  R  S  W  D  I  T
CGCACGCCGTCATCGGCCACTCGCTCGGCGAGATCACCGCCGCGTACGCC 2300
P  H  A  V  I  G  H  S  L  G  E  I  T  A  A  Y  A
GCCGGGATCCTGTCGCTCGACGACGCCTGCACCCTGATCACCACGCGTGC 2350
 A  G  I  L  S  L  D  D  A  C  T  L  I  T  T  R  A
CCGCCTCATGCACACGCTTCCGCCGCCCGGCGCCATGGTCACCGTGCTGA 2400
  R  L  M  H  T  L  P  P  P  G  A  M  V  T  V  L
CCAGCGAGGAGGAGGCCCGTCAGGCGCTGCGGCCGGGCGTGGAGATCGCC 2450
T  S  E  E  E  A  R  Q  A  L  R  P  G  V  E  I  A
GCGGTCTTCGGCCCGCACTCCGTCGTGCTCTCGGGCGACGAGGACGCCGT 2500
 A  V  F  G  P  H  S  V  V  L  S  G  D  E  D  A  V
GCTCGACGTCGCACAGCGGCTCGGCATCCACCACCGTCTGCCCGCGCCGC 2550
  L  D  V  A  Q  R  L  G  I  H  H  R  L  P  A  P
ACGCGGGCCACTCCGCGCACATGGAACCCGTGGCCGCCGAGCTGCTCGCC 2600
H  A  G  H  S  A  H  M  E  P  V  A  A  E  L  L  A
ACCACTCGCGAGCTCCGTTACGACCGGCCCCACACCGCCATCCCGAACGA 2650
 T  T  R  E  L  R  Y  D  R  P  H  T  A  I  P  N  D
CCCCACCACCGCCGAGTACTGGGCCGAGCAGGTCCGCAACCCCGTGCTGT 2700
  P  T  T  A  E  Y  W  A  E  Q  V  R  N  P  V  L
TCCACGCCCACACCCAGCGGTACCCCGACGCCGTGTTCGTCGAGATCGGC 2750
F  H  A  H  T  Q  R  Y  P  D  A  V  F  V  E  I  G
CCCGGCCAGGACCTCTCACCGCTGGTCGACGGCATCGCCCTGCAGAACGG 2800
 P  G  Q  D  L  S  P  L  V  D  G  I  A  L  Q  N  G
CACGGCGGACGAGGTGCACGCGCTGCACACCGCGCTCGCCCGCCTCTTCA 2850
  T  A  D  E  V  H  A  L  H  T  A  L  A  R  L  F
CACGCGGCGCCACGCTCGACTGGTCCCGCATCCTCGGCGGTGCTTCGCGG 2900
T  R  G  A  T  L  D  W  S  R  I  L  G  G  A  S  R
CACGACCCTGACGTCCCCTCGTACGCGTTCCAGCGGCGTCCCTACTGGAT 2950
 H  D  P  D  V  P  S  Y  A  F  Q  R  R  P  Y  W  I
```

```
CGAGTCGGCTCCCCCGGCCACGGCCGACTCGGGCCACCCCGTCCTCGGCA 3000
 E  S  A  P  P  A  T  A  D  S  G  H  P  V  L  G
CCGGAGTCGCCGTCGCCGGGTCGCCGGGCCGGGTGTTCACGGGTCCCGTG 3050
T  G  V  A  V  A  G  S  P  G  R  V  F  T  G  P  V
CCCGCCGGTGCGGACCGCGCGGTGTTCATCGCCGAACTGGCGCTCGCCGC 3100
 P  A  G  A  D  R  A  V  F  I  A  E  L  A  L  A  A
CGCCGACGCCACCGACTGCGCCACGGTCGAACAGCTCGACGTCACCTCCG 3150
 A  D  A  T  D  C  A  T  V  E  Q  L  D  V  T  S
TGCCCGGCGGATCCGCCCGCGGCAGGGCCACCGCGCAGACCTGGGTCGAT 3200
V  P  G  G  S  A  R  G  R  A  T  A  Q  T  W  V  D
GAACCCGCCGCCGACGGGCGGCGCCGCTTCACCGTCCACACCCGCGTCGG 3250
 E  P  A  A  D  G  R  R  R  F  T  V  H  T  R  V  G
CGACGCCCCGTGGACGCTGCACGCCGAGGGGGTTCTCCGCCCCGGCCGCG 3300
 D  A  P  W  T  L  H  A  E  G  V  L  R  P  G  R
TGCCCCAGCCCGAAGCCGTCGACACCGCCTGGCCCCCGCCGGGCGCGGTG 3350
V  P  Q  P  E  A  V  D  T  A  W  P  P  P  G  A  V
CCCGCGGACGGGCTGCCCGGGGCGTGGCGACGCGCGGACCAGGTCTTCGT 3400
 P  A  D  G  L  P  G  A  W  R  R  A  D  Q  V  F  V
CGAAGCCGAAGTCGACAGCCCTGACGGCTTCGTGGCACACCCCGACCTGC 3450
 E  A  E  V  D  S  P  D  G  F  V  A  H  P  D  L
TCGACGCGGTCTTCTCCGCGGTCGGCGACGGGAGCCGCCAGCCGACCGGA 3500
L  D  A  V  F  S  A  V  G  D  G  S  R  Q  P  T  G
TGGCGCGACCTCGCGGTGCACGCGTCGGACGCCACCGTGCTGCGCGCCTG 3550
 W  R  D  L  A  V  H  A  S  D  A  T  V  L  R  A  C
CCTCACCCGCCGCGACAGTGGTGTCGTGGAGCTCGCCGCCTTCGACGGTG 3600
 L  T  R  R  D  S  G  V  V  E  L  A  A  F  D  G
CCGGAATGCCGGTGCTCACCGCGGAGTCGGTGACGCTGGGCGAGGTCGCG 3650
A  G  M  P  V  L  T  A  E  S  V  T  L  G  E  V  A
TCGGCAGGCGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTT 3700
 S  A  G  G  S  D  E  S  D  G  L  L  R  L  E  W  L
GCCGGTGGCGGAGGCCCACTACGACGGTGCCGACGAGCTGCCCGAGGGCT 3750
 P  V  A  E  A  H  Y  D  G  A  D  E  L  P  E  G
ACACCCTCATCACCGCCACACACCCCGACGACCCCGACGACCCCACCAAC 3800
Y  T  L  I  T  A  T  H  P  D  D  P  D  D  P  T  N
CCCCACAACACACCCACACGCACCCACACACAAACCACACGCGTCCTCAC 3850
 P  H  N  T  P  T  R  T  H  T  Q  T  T  R  V  L  T
CGCCCTCCAACACCACCTCATCACCACCAACCACACCCTCATCGTCCACA 3900
 A  L  Q  H  H  L  I  T  T  N  H  T  L  I  V  H
CCACCACCGACCCCCCAGGCGCCGCCGTCACCGGCCTCACCCGCACCGCA 3950
T  T  T  D  P  P  G  A  A  V  T  G  L  T  R  T  A
CAAAACGAACACCCCGGCCGCATCCACCTCATCGAAACCCACCACCCCCA 4000
 Q  N  E  H  P  G  R  I  H  L  I  E  T  H  H  P  H
CACCCCACTCCCCCTCACCCAACTCACCACCCTCCACCAACCCCACCTAC 4050
 T  P  L  P  L  T  Q  L  T  T  L  H  Q  P  H  L
GCCTCACCAACAACACCCTCCACACCCCCCACCTCACCCCCATCACCACC 4100
R  L  T  N  N  T  L  H  T  P  H  L  T  P  I  T  T
CACCACAACACCACCACAACCACCCCCAACACCCCACCCCTCAACCCCAA 4150
 H  H  N  T  T  T  T  T  P  N  T  P  P  L  N  P  N
CCACGCCATCCTCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCG 4200
 H  A  I  L  I  T  G  G  S  G  T  L  A  G  I  L
CCCGCCACCTCAACCACCCCCACACCTACCTCCTCTCCCGCACACCACCA 4250
A  R  H  L  N  H  P  H  T  Y  L  L  S  R  T  P  P
CCCCCCACCACACCCGGCACCCACATCCCCTGCGACCTCACCGACCCCAC 4300
 P  P  T  T  P  G  T  H  I  P  C  D  L  T  D  P  T
```

```
CCAAATCACCCAAGCCCTCACCCACATACCACAACCCCTCACCGGCATCT 4350
  Q  I  T  Q  A  L  T  H  I  P  Q  P  L  T  G  I
TCCACACCGCCGCCACCCTCGACGACGCCACCCTCACCAACCTCACCCCC 4400
F  H  T  A  A  T  L  D  D  A  T  L  T  N  L  T  P
CAACACCTCACCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCT 4450
 Q  H  L  T  T  T  L  Q  P  K  A  D  A  A  W  H  L
CCACCACCACACCCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCA 4500
  H  H  H  T  Q  N  Q  P  L  T  H  F  V  L  Y  S
GCGCCGCCGCCACCCTCGGCAGCCCCGGCCAAGCCAACTACGCCGCCGCC 4550
S  A  A  A  T  L  G  S  P  G  Q  A  N  Y  A  A  A
AACGCCTTCCTCGACGCCCTCGCCACCCACCGCCACACCCAAGGACAACC 4600
 N  A  F  L  D  A  L  A  T  H  R  H  T  Q  G  Q  P
CGCCACCACCATCGCCTGGGGCATGTGGCACACCACCACCACACTCACCA 4650
  A  T  T  I  A  W  G  M  W  H  T  T  T  T  L  T
GCCAACTCACCGACAGCGACCGCGACCGCATCCGCCGCGGCGGCTTCCTG 4700
S  Q  L  T  D  S  D  R  D  R  I  R  R  G  G  F  L
CCGATCTCGGACGACGAGGGCATGC
 P  I  S  D  D  E  G  M
```

The *Avr*II-*Xho*I hybrid FK-506 PKS module 8 containing the AT domain of module 12 of rapamycin is shown below.

```
GCATGCGGCTGTACGAGGCGGCACGGCGCACCGGAAGTCCCGTGGTGGTG 50
  M  R  L  Y  E  A  A  R  R  T  G  S  P  V  V  V
GCGGCCGCGCTCGACGACGCGCCGGACGTGCCGCTGCTGCGCGGGCTGCG 100
 A  A  A  L  D  D  A  P  D  V  P  L  L  R  G  L  R
GCGTACGACCGTCCGGCGTGCCGCCGTCCGGGAACGCTCTCTCGCCGACC 150
  R  T  T  V  R  R  A  A  V  R  E  R  S  L  A  D
GCTCGCCGTGCTGCCCGACGACGAGCGCGCCGACGCCTCCCTCGCGTTCG 200
R  S  P  C  C  P  T  T  S  A  P  T  P  P  S  R  S
TCCTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCGCCGAAGACAT 250
 S  W  N  S  T  A  T  V  L  G  H  L  G  A  E  D  I
CCCGGCGACGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300
  P  A  T  T  T  F  K  E  L  G  I  D  S  L  T  A
TCCAGCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350
V  Q  L  R  N  A  L  T  T  A  T  G  V  R  L  N  A
ACAGCGGTCTTCGACTTTCCGACGCCGCGCGCGCTCGCCGCGAGACTCGG 400
 T  A  V  F  D  F  P  T  P  R  A  L  A  A  R  L  G
CGACGAGCTGGCCGGTACCCGCGCGCCCGTCGCGGCCCGGACCGCGGCCA 450
  D  E  L  A  G  T  R  A  P  V  A  A  R  T  A  A
CCGCGGCCGCGCACGACGAACCGCTGGCGATCGTGGGCATGGCCTGCCGT 500
T  A  A  A  H  D  E  P  L  A  I  V  G  M  A  C  R
CTGCCGGGCGGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550
 L  P  G  G  V  A  S  P  Q  E  L  W  R  L  V  A  S
CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG 600
  G  T  D  A  I  T  E  F  P  A  D  R  G  W  D  V
ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650
D  A  L  Y  D  P  D  P  D  A  I  G  K  T  F  V  R
CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGGCGTTCTTCGG 700
 H  G  G  F  L  D  G  A  T  G  F  D  A  A  F  F  G
GATCAGCCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750
  I  S  P  R  E  A  L  A  M  D  P  Q  Q  R  V  L
TGGAGACGTCCTGGGAGGCGTTCGAAAGCGCGGGCATCACCCCGGACGCG 800
```

```
L  E  T  S  W  E  A  F  E  S  A  G  I  T  P  D  A
GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850
 A  R  G  S  D  T  G  V  F  I  G  A  F  S  Y  G  Y
CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900
  G  T  G  A  D  T  N  G  F  G  A  T  G  S  Q  T
GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
S  V  L  S  G  R  L  S  Y  F  Y  G  L  E  G  P  S
GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000
 V  T  V  D  T  A  C  S  S  S  L  V  A  L  H  Q  A
AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050
  G  Q  S  L  R  S  G  E  C  S  L  A  L  V  G  G
TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100
V  T  V  M  A  S  P  G  G  F  V  E  F  S  R  Q  R
GGGCTCGCGCCGGACGGGCGGGCGAAGGCGTTCGGCGCGGGCGCGGACGG 1150
 G  L  A  P  D  G  R  A  K  A  F  G  A  G  A  D  G
TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG 1200
  T  S  F  A  E  G  A  G  A  L  V  V  E  R  L  S
ACGCGGAGCGCCACGGCCACACCGTCCTCGCCCTCGTACGCGGCTCCGCG 1250
D  A  E  R  H  G  H  T  V  L  A  L  V  R  G  S  A
GCTAACTCCGACGGCGCGTCGAACGGTCTGTCGGCGCCGAACGGCCCCTC 1300
 A  N  S  D  G  A  S  N  G  L  S  A  P  N  G  P  S
CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350
  Q  E  R  V  I  H  Q  A  L  A  N  A  K  L  T  P
CCGATGTCGACGCGGTCGAGGCGCACGGCACCGGCACCCGCCTCGGCGAC 1400
A  D  V  D  A  V  E  A  H  G  T  G  T  R  L  G  D
CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGCGAC 1450
 P  I  E  A  Q  A  L  L  A  T  Y  G  Q  D  R  A  T
GCCCCTGCTGCTCGGCTCGCTGAAGTCGAACATCGGGCACGCCCAGGCCG 1500
  P  L  L  L  G  S  L  K  S  N  I  G  H  A  Q  A
CGTCAGGGGTCGCCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGGG 1550
A  S  G  V  A  G  I  I  K  M  V  Q  A  I  R  H  G
GAACTGCCGCCGACACTGCACGCGGACGAGCCGTCGCCGCACGTCGACTG 1600
 E  L  P  P  T  L  H  A  D  E  P  S  P  H  V  D  W
GACGGCCGGTGCCGTCGAGCTCCTGACGTCGGCCCGGCCGTGGCCGGGGA 1650
  T  A  G  A  V  E  L  L  T  S  A  R  P  W  P  G
CCGGTCGCCCTAGGCGGGCAGGCGTGTCGTCCTTCGGGATCAGTGGCACC 1700
T  G  R  P  R  R  A  G  V  S  S  F  G  I  S  G  T
AACGCCCACGTCATCCTGGAAAGCGCACCCCCCACTCAGCCTGCGGACAA 1750
 N  A  H  V  I  L  E  S  A  P  P  T  Q  P  A  D  N
CGCGGTGATCGAGCGGGCACCGGAGTGGGTGCCGTTGGTGATTTCGGCCA 1800
  A  V  I  E  R  A  P  E  W  V  P  L  V  I  S  A
GGACCCAGTCGGCTTTGACTGAGCACGAGGGCCGGTTGCGTGCGTATCTG 1850
R  T  Q  S  A  L  T  E  H  E  G  R  L  R  A  Y  L
GCGGCGTCGCCCGGGGTGGATATGCGGGCTGTGGCATCGACGCTGGCGAT 1900
 A  A  S  P  G  V  D  M  R  A  V  A  S  T  L  A  M
GACACGGTCGGTGTTCGAGCACCGTGCCGTGCTGCTGGGAGATGACACCG 1950
  T  R  S  V  F  E  H  R  A  V  L  L  G  D  D  T
TCACCGGCACCGCTGTGTCTGACCCTCGGGCGGTGTTCGTCTTCCCGGGA 2000
V  T  G  T  A  V  S  D  P  R  A  V  F  V  F  P  G
CAGGGGTCGCAGCGTGCTGGCATGGGTGAGGAACTGGCCGCCGCGTTCCC 2050
 Q  G  S  Q  R  A  G  M  G  E  E  L  A  A  A  F  P
CGTCTTCGCGCGGATCCATCAGCAGGTGTGGGACCTGCTCGATGTGCCCG 2100
  V  F  A  R  I  H  Q  Q  V  W  D  L  L  D  V  P
ATCTGGAGGTGAACGAGACCGGTTACGCCCAGCCGGCCCTGTTCGCAATG 2150
```

```
D  L  E  V  N  E  T  G  Y  A  Q  P  A  L  F  A  M
CAGGTGGCTCTGTTCGGGCTGCTGGAATCGTGGGGTGTACGACCGGACGC 2200
 Q  V  A  L  F  G  L  L  E  S  W  G  V  R  P  D  A
GGTGATCGGCCATTCGGTGGGTGAGCTTGCGGCTGCGTATGTGTCCGGGG 2250
  V  I  G  H  S  V  G  E  L  A  A  A  Y  V  S  G
TGTGGTCGTTGGAGGATGCCTGCACTTTGGTGTCGGCGCGGGCTCGTCTG 2300
V  W  S  L  E  D  A  C  T  L  V  S  A  R  A  R  L
ATGCAGGCTCTGCCCGCGGGTGGGGTGATGGTCGCTGTCCCGGTCTCGGA 2350
 M  Q  A  L  P  A  G  G  V  M  V  A  V  P  V  S  E
GGATGAGGCCCGGGCCGTGCTGGGTGAGGGTGTGGAGATCGCCGCGGTCA 2400
  D  E  A  R  A  V  L  G  E  G  V  E  I  A  A  V
ACGGCCCGTCGTCGGTGGTTCTCTCCGGTGATGAGGCCGCCGTGCTGCAG 2450
N  G  P  S  S  V  V  L  S  G  D  E  A  A  V  L  Q
GCCGCGGAGGGGCTGGGGAAGTGGACGCGGCTGGCGACCAGCCACGCGTT 2500
 A  A  E  G  L  G  K  W  T  R  L  A  T  S  H  A  F
CCATTCCGCCCGTATGGAACCCATGCTGGAGGAGTTCCGGGCGGTCGCCG 2550
  H  S  A  R  M  E  P  M  L  E  E  F  R  A  V  A
AAGGCCTGACCTACCGGACGCCGCAGGTCTCCATGGCCGTTGGTGATCAG 2600
E  G  L  T  Y  R  T  P  Q  V  S  M  A  V  G  D  Q
GTGACCACCGCTGAGTACTGGGTGCGGCAGGTCCGGGACACGGTCCGGTT 2650
 V  T  T  A  E  Y  W  V  R  Q  V  R  D  T  V  R  F
CGGCGAGCAGGTGGCCTCGTACGAGGACGCCGTGTTCGTCGAGCTGGGTG 2700
  G  E  Q  V  A  S  Y  E  D  A  V  F  V  E  L  G
CCGACCGGTCACTGGCCCGCCTGGTCGACGGTGTCGCGATGCTGCACGGC 2750
A  D  R  S  L  A  R  L  V  D  G  V  A  M  L  H  G
GACCACGAAATCCAGGCCGCGATCGGCGCCCTGGCCCACCTGTATGTCAA 2800
 D  H  E  I  Q  A  A  I  G  A  L  A  H  L  Y  V  N
CGGCGTCACGGTCGACTGGCCCGCGCTCCTGGGCGATGCTCCGGCAACAC 2850
  G  V  T  V  D  W  P  A  L  L  G  D  A  P  A  T
GGGTGCTGGACCTTCCGACATACGCCTTCCAGCACCAGCGCTACTGGCTC 2900
R  V  L  D  L  P  T  Y  A  F  Q  H  Q  R  Y  W  L
GAGTCGGCTCCCCCGGCCACGGCCGACTCGGGCCACCCCGTCCTCGGCAC 2950
 E  S  A  P  P  A  T  A  D  S  G  H  P  V  L  G  T
CGGAGTCGCCGTCGCCGGGTCGCCGGGCCGGGTGTTCACGGGTCCCGTGC 3000
  G  V  A  V  A  G  S  P  G  R  V  F  T  G  P  V
CCGCCGGTGCGGACCGCGCGGTGTTCATCGCCGAACTGGCGCTCGCCGCC 3050
P  A  G  A  D  R  A  V  F  I  A  E  L  A  L  A  A
GCCGACGCCACCGACTGCGCCACGGTCGAACAGCTCGACGTCACCTCCGT 3100
 A  D  A  T  D  C  A  T  V  E  Q  L  D  V  T  S  V
GCCCGGCGGATCCGCCCGCGGCAGGGCCACCGCGCAGACCTGGGTCGATG 3150
  P  G  G  S  A  R  G  R  A  T  A  Q  T  W  V  D
AACCCGCCGCCGACGGGCGGCGCCGCTTCACCGTCCACACCCGCGTCGGC 3200
E  P  A  A  D  G  R  R  R  F  T  V  H  T  R  V  G
GACGCCCCGTGGACGCTGCACGCCGAGGGGGTTCTCCGCCCCGGCCGCGT 3250
 D  A  P  W  T  L  H  A  E  G  V  L  R  P  G  R  V
GCCCCAGCCCGAAGCCGTCGACACCGCCTGGCCCCCGCCGGGCGCGGTGC 3300
  P  Q  P  E  A  V  D  T  A  W  P  P  P  G  A  V
CCGCGGACGGGCTGCCCGGGGCGTGGCGACGCGCGGACCAGGTCTTCGTC 3350
P  A  D  G  L  P  G  A  W  R  R  A  D  Q  V  F  V
GAAGCCGAAGTCGACAGCCCTGACGGCTTCGTGGCACACCCCGACCTGCT 3400
 E  A  E  V  D  S  P  D  G  F  V  A  H  P  D  L  L
CGACGCGGTCTTCTCCGCGGTCGGCGACGGGAGCCGCCAGCCGACCGGAT 3450
  D  A  V  F  S  A  V  G  D  G  S  R  Q  P  T  G
GGCGCGACCTCGCGGTGCACGCGTCGGACGCCACCGTGCTGCGCGCCTGC 3500
```

```
W  R  D  L  A  V  H  A  S  D  A  T  V  L  R  A  C
CTCACCCGCCGCGACAGTGGTGTCGTGGAGCTCGCCGCCTTCGACGGTGC 3550
 L  T  R  R  D  S  G  V  V  E  L  A  A  F  D  G  A
CGGAATGCCGGTGCTCACCGCGGAGTCGGTGACGCTGGGCGAGGTCGCGT 3600
  G  M  P  V  L  T  A  E  S  V  T  L  G  E  V  A
CGGCAGGCGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTG 3650
S  A  G  G  S  D  E  S  D  G  L  L  R  L  E  W  L
CCGGTGGCGGAGGCCCACTACGACGGTGCCGACGAGCTGCCCGAGGGCTA 3700
 P  V  A  E  A  H  Y  D  G  A  D  E  L  P  E  G  Y
CACCCTCATCACCGCCACACACCCCGACGACCCCGACGACCCCACCAACC 3750
  T  L  I  T  A  T  H  P  D  D  P  D  D  P  T  N
CCCACAACACACCCACACGCACCCACACACAAACCACACGCGTCCTCACC 3800
P  H  N  T  P  T  R  T  H  T  Q  T  T  R  V  L  T
GCCCTCCAACACCACCTCATCACCACCAACCACACCCTCATCGTCCACAC 3850
 A  L  Q  H  H  L  I  T  T  N  H  T  L  I  V  H  T
CACCACCGACCCCCCAGGCGCCGCCGTCACCGGCCTCACCCGCACCGCAC 3900
  T  T  D  P  P  G  A  A  V  T  G  L  T  R  T  A
AAAACGAACACCCCGGCCGCATCCACCTCATCGAAACCCACCACCCCCAC 3950
Q  N  E  H  P  G  R  I  H  L  I  E  T  H  H  P  H
ACCCCACTCCCCCTCACCCAACTCACCACCCTCCACCAACCCCACCTACG 4000
 T  P  L  P  L  T  Q  L  T  T  L  H  Q  P  H  L  R
CCTCACCAACAACACCCTCCACACCCCCCACCTCACCCCCATCACCACCC 4050
  L  T  N  N  T  L  H  T  P  H  L  T  P  I  T  T
ACCACAACACCACCACAACCACCCCCAACACCCCACCCCTCAACCCCAAC 4100
H  H  N  T  T  T  T  T  P  N  T  P  P  L  N  P  N
CACGCCATCCTCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGC 4150
 H  A  I  L  I  T  G  G  S  G  T  L  A  G  I  L  A
CCGCCACCTCAACCACCCCCACACCTACCTCCTCTCCCGCACACCACCAC 4200
  R  H  L  N  H  P  H  T  Y  L  L  S  R  T  P  P
CCCCCACCACACCCGGCACCCACATCCCCTGCGACCTCACCGACCCCACC 4250
P  P  T  T  P  G  T  H  I  P  C  D  L  T  D  P  T
CAAATCACCCAAGCCCTCACCCACATACCACAACCCCTCACCGGCATCTT 4300
 Q  I  T  Q  A  L  T  H  I  P  Q  P  L  T  G  I  F
CCACACCGCCGCCACCCTCGACGACGCCACCCTCACCAACCTCACCCCCC 4350
  H  T  A  A  T  L  D  D  A  T  L  T  N  L  T  P
AACACCTCACCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCTC 4400
Q  H  L  T  T  T  L  Q  P  K  A  D  A  A  W  H  L
CACCACCACACCCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCAG 4450
 H  H  H  T  Q  N  Q  P  L  T  H  F  V  L  Y  S  S
CGCCGCCGCCACCCTCGGCAGCCCCGGCCAAGCCAACTACGCCGCCGCCA 4500
  A  A  A  T  L  G  S  P  G  Q  A  N  Y  A  A  A
ACGCCTTCCTCGACGCCCTCGCCACCCACCGCCACACCCAAGGACAACCC 4550
N  A  F  L  D  A  L  A  T  H  R  H  T  Q  G  Q  P
GCCACCACCATCGCCTGGGGCATGTGGCACACCACCACCACACTCACCAG 4600
 A  T  T  I  A  W  G  M  W  H  T  T  T  T  L  T  S
CCAACTCACCGACAGCGACCGCGACCGCATCCGCCGCGGCGGCTTCCTGC 4650
  Q  L  T  D  S  D  R  D  R  I  R  R  G  G  F  L
CGATCTCGGACGACGAGGGCATGC
P  I  S  D  D  E  G  M
```

The *Avr*II-*Xho*I hybrid FK-506 PKS module 8 containing the AT domain of module 13 of rapamycin is shown below.

GCATGCGGCTGTACGAGGCGGCACGGCGCACCGGAAGTCCCGTGGTGGTG 50
M R L Y E A A R R T G S P V V V
GCGGCCGCGCTCGACGACGCGCCGGACGTGCCGCTGCTGCGCGGGCTGCG 100
A A A L D D A P D V P L L R G L R
GCGTACGACCGTCCGGCGTGCCGCCGTCCGGGAACGCTCTCTCGCCGACC 150
R T T V R R A A V R E R S L A D
GCTCGCCGTGCTGCCCGACGACGAGCGCGCCGACGCCTCCCTCGCGTTCG 200
R S P C C P T T S A P T P P S R S
TCCTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCGCCGAAGACAT 250
S W N S T A T V L G H L G A E D I
CCCGGCGACGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300
P A T T T F K E L G I D S L T A
TCCAGCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350
V Q L R N A L T T A T G V R L N A
ACAGCGGTCTTCGACTTTCCGACGCCGCGCGCGCTCGCCGCGAGACTCGG 400
T A V F D F P T P R A L A A R L G
CGACGAGCTGGCCGGTACCCGCGCGCCCGTCGCGGCCCGGACCGCGGCCA 450
D E L A G T R A P V A A R T A A
CCGCGGCCGCGCACGACGAACCGCTGGCGATCGTGGGCATGGCCTGCCGT 500
T A A A H D E P L A I V G M A C R
CTGCCGGGCGGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550
L P G G V A S P Q E L W R L V A S
CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG 600
G T D A I T E F P A D R G W D V
ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650
D A L Y D P D P D A I G K T F V R
CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGGCGTTCTTCGG 700
H G G F L D G A T G F D A A F F G
GATCAGCCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750
I S P R E A L A M D P Q Q R V L
TGGAGACGTCCTGGGAGGCGTTCGAAAGCGCGGGCATCACCCCGGACGCG 800
L E T S W E A F E S A G I T P D A
GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850
A R G S D T G V F I G A F S Y G Y
CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900
G T G A D T N G F G A T G S Q T
GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
S V L S G R L S Y F Y G L E G P S
GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000
V T V D T A C S S S L V A L H Q A
AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050
G Q S L R S G E C S L A L V G G
TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100
V T V M A S P G G F V E F S R Q R
GGGCTCGCGCCGGACGGGCGGGCGAAGGCGTTCGGCGCGGGCGCGGACGG 1150
G L A P D G R A K A F G A G A D G
TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG 1200
T S F A E G A G A L V V E R L S
ACGCGGAGCGCCACGGCCACACCGTCCTCGCCCTCGTACGCGGCTCCGCG 1250
D A E R H G H T V L A L V R G S A
GCTAACTCCGACGGCGCGTCGAACGGTCTGTCGGCGCCGAACGGCCCCTC 1300
A N S D G A S N G L S A P N G P S
CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350
Q E R V I H Q A L A N A K L T P

```
CCGATGTCGACGCGGTCGAGGCGCACGGCACCGGCACCCGCCTCGGCGAC 1400
A  D  V  D  A  V  E  A  H  G  T  G  T  R  L  G  D
CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGCGAC 1450
 P  I  E  A  Q  A  L  L  A  T  Y  G  Q  D  R  A  T
GCCCCTGCTGCTCGGCTCGCTGAAGTCGAACATCGGGCACGCCCAGGCCG 1500
  P  L  L  G  S  L  K  S  N  I  G  H  A  Q  A
CGTCAGGGGTCGCCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGGG 1550
A  S  G  V  A  G  I  I  K  M  V  Q  A  I  R  H  G
GAACTGCCGCCGACACTGCACGCGGACGAGCCGTCGCCGCACGTCGACTG 1600
 E  L  P  P  T  L  H  A  D  E  P  S  P  H  V  D  W
GACGGCCGGTGCCGTCGAGCTCCTGACGTCGGCCCGGCCGTGGCCGGGGA 1650
  T  A  G  A  V  E  L  L  T  S  A  R  P  W  P  G
CCGGTCGCCCTAGGCGGGCGGGCGTGTCGTCCTTCGGAGTCAGCGGCACC 1700
T  G  R  P  R  R  A  G  V  S  S  F  G  V  S  G  T
AACGCCCACGTCATCCTGGAGAGCGCACCCCCCGCTCAGCCCGCGGAGGA 1750
 N  A  H  V  I  L  E  S  A  P  P  A  Q  P  A  E  E
GGCGCAGCCTGTTGAGACGCCGGTGGTGGCCTCGGATGTGCTGCCGCTGG 1800
  A  Q  P  V  E  T  P  V  V  A  S  D  V  L  P  L
TGATATCGGCCAAGACCCAGCCCGCCCTGACCGAACACGAAGACCGGCTG 1850
V  I  S  A  K  T  Q  P  A  L  T  E  H  E  D  R  L
CGCGCCTACCTGGCGGCGTCGCCCGGGGCGGATATACGGGCTGTGGCATC 1900
 R  A  Y  L  A  A  S  P  G  A  D  I  R  A  V  A  S
GACGCTGGCGGTGACACGGTCGGTGTTCGAGCACCGCGCCGTACTCCTTG 1950
  T  L  A  V  T  R  S  V  F  E  H  R  A  V  L  L
GAGATGACACCGTCACCGGCACCGCGGTGACCGACCCCAGGATCGTGTTT 2000
G  D  D  T  V  T  G  T  A  V  T  D  P  R  I  V  F
GTCTTTCCCGGGCAGGGGTGGCAGTGGCTGGGGATGGGCAGTGCACTGCG 2050
 V  F  P  G  Q  G  W  Q  W  L  G  M  G  S  A  L  R
CGATTCGTCGGTGGTGTTCGCCGAGCGGATGGCCGAGTGTGCGGCGGCGT 2100
  D  S  S  V  V  F  A  E  R  M  A  E  C  A  A  A
TGCGCGAGTTCGTGGACTGGGATCTGTTCACGGTTCTGGATGATCCGGCG 2150
L  R  E  F  V  D  W  D  L  F  T  V  L  D  D  P  A
GTGGTGGACCGGGTTGATGTGGTCCAGCCCGCTTCCTGGGCGATGATGGT 2200
 V  V  D  R  V  D  V  V  Q  P  A  S  W  A  M  M  V
TTCCCTGGCCGCGGTGTGGCAGGCGGCCGGTGTGCGGCCGGATGCGGTGA 2250
  S  L  A  A  V  W  Q  A  A  G  V  R  P  D  A  V
TCGGCCATTCGCAGGGTGAGATCGCCGCAGCTTGTGTGGCGGGTGCGGTG 2300
I  G  H  S  Q  G  E  I  A  A  A  C  V  A  G  A  V
TCACTACGCGATGCCGCCCGGATCGTGACCTTGCGCAGCCAGGCGATCGC 2350
 S  L  R  D  A  A  R  I  V  T  L  R  S  Q  A  I  A
CCGGGGCCTGGCGGGCCGGGGCGCGATGGCATCCGTCGCCCTGCCCGCGC 2400
  R  G  L  A  G  R  G  A  M  A  S  V  A  L  P  A
AGGATGTCGAGCTGGTCGACGGGGCCTGGATCGCCGCCCACAACGGGCCC 2450
Q  D  V  E  L  V  D  G  A  W  I  A  A  H  N  G  P
GCCTCCACCGTGATCGCGGGCACCCCGGAAGCGGTCGACCATGTCCTCAC 2500
 A  S  T  V  I  A  G  T  P  E  A  V  D  H  V  L  T
CGCTCATGAGGCACAAGGGGTGCGGGTGCGGCGGATCACCGTCGACTATG 2550
  A  H  E  A  Q  G  V  R  V  R  R  I  T  V  D  Y
CCTCGCACACCCCGCACGTCGAGCTGATCCGCGACGAACTACTCGACATC 2600
A  S  H  T  P  H  V  E  L  I  R  D  E  L  L  D  I
ACTAGCGACAGCAGCTCGCAGACCCCGCTCGTGCCGTGGCTGTCGACCGT 2650
 T  S  D  S  S  S  Q  T  P  L  V  P  W  L  S  T  V
GGACGGCACCTGGGTCGACAGCCCGCTGGACGGGGAGTACTGGTACCGGA 2700
  D  G  T  W  V  D  S  P  L  D  G  E  Y  W  Y  R
```

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ACCTGCGTGAACCGGTCGGTTTCCACCCCGCCGTCAGCCAGTTGCAGGCC 2750
N  L  R  E  P  V  G  F  H  P  A  V  S  Q  L  Q  A
CAGGGCGACACCGTGTTCGTCGAGGTCAGCGCCAGCCCGGTGTTGTTGCA 2800
 Q  G  D  T  V  F  V  E  V  S  A  S  P  V  L  L  Q
GGCGATGGACGACGATGTCGTCACGGTTGCCACGCTGCGTCGTGACGACG 2850
  A  M  D  D  D  V  V  T  V  A  T  L  R  R  D  D
GCGACGCCACCCGGATGCTCACCGCCCTGGCACAGGCCTATGTCCACGGC 2900
G  D  A  T  R  M  L  T  A  L  A  Q  A  Y  V  H  G
GTCACCGTCGACTGGCCCGCCATCCTCGGCACCACCACAACCCGGGTACT 2950
 V  T  V  D  W  P  A  I  L  G  T  T  T  T  R  V  L
GGACCTTCCGACCTACGCCTTCCAACACCAGCGGTACTGGCTCGAGTCGG 3000
  D  L  P  T  Y  A  F  Q  H  Q  R  Y  W  L  E  S
CTCCCCCGGCCACGGCCGACTCGGGCCACCCCGTCCTCGGCACCGGAGTC 3050
A  P  P  A  T  A  D  S  G  H  P  V  L  G  T  G  V
GCCGTCGCCGGGTCGCCGGGCCGGGTGTTCACGGGTCCCGTGCCCGCCGG 3100
 A  V  A  G  S  P  G  R  V  F  T  G  P  V  P  A  G
TGCGGACCGCGCGGTGTTCATCGCCGAACTGGCGCTCGCCGCCGCCGACG 3150
  A  D  R  A  V  F  I  A  E  L  A  L  A  A  A  D
CCACCGACTGCGCCACGGTCGAACAGCTCGACGTCACCTCCGTGCCCGGC 3200
A  T  D  C  A  T  V  E  Q  L  D  V  T  S  V  P  G
GGATCCGCCCGCGGCAGGGCCACCGCGCAGACCTGGGTCGATGAACCCGC 3250
 G  S  A  R  G  R  A  T  A  Q  T  W  V  D  E  P  A
CGCCGACGGGCGGCGCCCGCTTCACCGTCCACACCCGCGTCGGCGACGCCC 3300
  A  D  G  R  R  R  F  T  V  H  T  R  V  G  D  A
CGTGGACGCTGCACGCCGAGGGGGTTCTCCGCCCCGGCCGCGTGCCCCAG 3350
P  W  T  L  H  A  E  G  V  L  R  P  G  R  V  P  Q
CCCGAAGCCGTCGACACCGCCTGGCCCCCGCCGGGCGCGGTGCCCGCGGA 3400
 P  E  A  V  D  T  A  W  P  P  P  G  A  V  P  A  D
CGGGCTGCCCGGGGCGTGGCGACGCGCGGACCAGGTCTTCGTCGAAGCCG 3450
  G  L  P  G  A  W  R  R  A  D  Q  V  F  V  E  A
AAGTCGACAGCCCTGACGGCTTCGTGGCACACCCCGACCTGCTCGACGCG 3500
E  V  D  S  P  D  G  F  V  A  H  P  D  L  L  D  A
GTCTTCTCCGCGGTCGGCGACGGGAGCCGCCAGCCGACCGGATGGCGCGA 3550
 V  F  S  A  V  G  D  G  S  R  Q  P  T  G  W  R  D
CCTCGCGGTGCACGCGTCGGACGCCACCGTGCTGCGCGCCTGCCTCACCC 3600
  L  A  V  H  A  S  D  A  T  V  L  R  A  C  L  T
GCCGCGACAGTGGTGTCGTGGAGCTCGCCGCCTTCGACGGTGCCGGAATG 3650
R  R  D  S  G  V  V  E  L  A  A  F  D  G  A  G  M
CCGGTGCTCACCGCGGAGTCGGTGACGCTGGGCGAGGTCGCGTCGGCAGG 3700
 P  V  L  T  A  E  S  V  T  L  G  E  V  A  S  A  G
CGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTGCCGGTGG 3750
  G  S  D  E  S  D  G  L  L  R  L  E  W  L  P  V
CGGAGGCCCACTACGACGGTGCCGACGAGCTGCCCGAGGGCTACACCCTC 3800
A  E  A  H  Y  D  G  A  D  E  L  P  E  G  Y  T  L
ATCACCGCCACACACCCCGACGACCCCGACGACCCCACCAACCCCCACAA 3850
 I  T  A  T  H  P  D  D  P  D  D  P  T  N  P  H  N
CACACCCACACGCACCCACACACAAACCACACGCGTCCTCACCGCCCTCC 3900
  T  P  T  R  T  H  T  Q  T  T  R  V  L  T  A  L
AACACCACCTCATCACCACCAACCACACCCTCATCGTCCACACCACCACC 3950
Q  H  H  L  I  T  T  N  H  T  L  I  V  H  T  T  T
GACCCCCCAGGCGCCGCCGTCACCGGCCTCACCCGCACCGCACAAAACGA 4000
 D  P  P  G  A  A  V  T  G  L  T  R  T  A  Q  N  E
ACACCCCGGCCGCATCCACCTCATCGAAACCCACCACCCCCACACCCCAC 4050
  H  P  G  R  I  H  L  I  E  T  H  H  P  H  T  P
```

```
TCCCCCTCACCCAACTCACCACCCTCCACCAACCCCACCTACGCCTCACC 4100
L  P  L  T  Q  L  T  T  L  H  Q  P  H  L  R  L  T
AACAACACCCTCCACACCCCCCACCTCACCCCCATCACCACCCACCACAA 4150
 N  N  T  L  H  T  P  H  L  T  P  I  T  T  H  H  N
CACCACCACAACCACCCCCAACACCCCACCCCTCAACCCCAACCACGCCA 4200
  T  T  T  T  T  P  N  T  P  P  L  N  P  N  H  A
TCCTCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCCGCCAC 4250
I  L  I  T  G  G  S  G  T  L  A  G  I  L  A  R  H
CTCAACCACCCCCACACCTACCTCCTCTCCCGCACACCACCACCCCCCAC 4300
 L  N  H  P  H  T  Y  L  L  S  R  T  P  P  P  P  T
CACACCCGGCACCCACATCCCCTGCGACCTCACCGACCCCACCCAAATCA 4350
  T  P  G  T  H  I  P  C  D  L  T  D  P  T  Q  I
CCCAAGCCCTCACCCACATACCACAACCCCTCACCGGCATCTTCCACACC 4400
T  Q  A  L  T  H  I  P  Q  P  L  T  G  I  F  H  T
GCCGCCACCCTCGACGACGCCACCCTCACCAACCTCACCCCCCAACACCT 4450
 A  A  T  L  D  D  A  T  L  T  N  L  T  P  Q  H  L
CACCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCTCCACCACC 4500
  T  T  T  L  Q  P  K  A  D  A  A  W  H  L  H  H
ACACCCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCAGCGCCGCC 4550
H  T  Q  N  Q  P  L  T  H  F  V  L  Y  S  S  A  A
GCCACCCTCGGCAGCCCCGGCCAAGCCAACTACGCCGCCGCCAACGCCTT 4600
 A  T  L  G  S  P  G  Q  A  N  Y  A  A  A  N  A  F
CCTCGACGCCCTCGCCACCCACCGCCACACCCAAGGACAACCCGCCACCA 4600
  L  D  A  L  A  T  H  R  H  T  Q  G  Q  P  A  T
CCATCGCCTGGGGCATGTGGCACACCACCACCACACTCACCAGCCAACTC 4700
T  I  A  W  G  M  W  H  T  T  T  T  L  T  S  Q  L
ACCGACAGCGACCGCGACCGCATCCGCCGCGGCGGCTTCCTGCCGATCTC 4750
 T  D  S  D  R  D  R  I  R  R  G  G  F  L  P  I  S
GGACGACGAGGGCATGC
  D  D  E  G  M
```

The *Nhe*I-*Xho*I hybrid FK-506 PKS module 8 containing the AT domain of module 12 of rapamycin is shown below.

```
GCATGCGGCTGTACGAGGCGGCACGGCGCACCGGAAGTCCCGTGGTGGTG 50
   M  R  L  Y  E  A  A  R  R  T  G  S  P  V  V  V
GCGGCCGCGCTCGACGACGCGCCGGACGTGCCGCTGCTGCGCGGGCTGCG 100
 A  A  A  L  D  D  A  P  D  V  P  L  L  R  G  L  R
GCGTACGACCGTCCGGCGTGCCGCCGTCCGGGAACGCTCTCTCGCCGACC 150
  R  T  T  V  R  R  A  A  V  R  E  R  S  L  A  D
GCTCGCCGTGCTGCCCGACGACGAGCGCGCCGACGCCTCCCTCGCGTTCG 200
R  S  P  C  C  P  T  T  S  A  P  T  P  P  S  R  S
TCCTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCGCCGAAGACAT 250
 S  W  N  S  T  A  T  V  L  G  H  L  G  A  E  D  I
CCCGGCGACGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300
  P  A  T  T  T  F  K  E  L  G  I  D  S  L  T  A
TCCAGCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350
V  Q  L  R  N  A  L  T  T  A  T  G  V  R  L  N  A
ACAGCGGTCTTCGACTTTCCGACGCCGCGCGCGCTCGCCGCGAGACTCGG 400
 T  A  V  F  D  F  P  T  P  R  A  L  A  A  R  L  G
CGACGAGCTGGCCGGTACCCGCGCGCCCGTCGCGGCCCGGACCGCGGCCA 450
  D  E  L  A  G  T  R  A  P  V  A  A  R  T  A  A
CCGCGGCCGCGCACGACGAACCGCTGGCGATCGTGGGCATGGCCTGCCGT 500
```

```
T  A  A  A  H  D  E  P  L  A  I  V  G  M  A  C  R
CTGCCGGGCGGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550
 L  P  G  G  V  A  S  P  Q  E  L  W  R  L  V  A  S
CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG 600
  G  T  D  A  I  T  E  F  P  A  D  R  G  W  D  V
ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650
D  A  L  Y  D  P  D  P  D  A  I  G  K  T  F  V  R
CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGGCGTTCTTCGG 700
 H  G  G  F  L  D  G  A  T  G  F  D  A  A  F  F  G
GATCAGCCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750
  I  S  P  R  E  A  L  A  M  D  P  Q  Q  R  V  L
TGGAGACGTCCTGGGAGGCGTTCGAAAGCGCGGGCATCACCCCGGACGCG 800
L  E  T  S  W  E  A  F  E  S  A  G  I  T  P  D  A
GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850
 A  R  G  S  D  T  G  V  F  I  G  A  F  S  Y  G  Y
CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900
  G  T  G  A  D  T  N  G  F  G  A  T  G  S  Q  T
GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
S  V  L  S  G  R  L  S  Y  F  Y  G  L  E  G  P  S
GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000
 V  T  V  D  T  A  C  S  S  S  L  V  A  L  H  Q  A
AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050
  G  Q  S  L  R  S  G  E  C  S  L  A  L  V  G  G
TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100
V  T  V  M  A  S  P  G  G  F  V  E  F  S  R  Q  R
GGGCTCGCGCCGGACGGGCGGGCGAAGGCGTTCGGCGCGGGCGCGGACGG 1150
 G  L  A  P  D  G  R  A  K  A  F  G  A  G  A  D  G
TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG 1200
  T  S  F  A  E  G  A  G  A  L  V  V  E  R  L  S
ACGCGGAGCGCCACGGCCACACCGTCCTCGCCCTCGTACGCGGCTCCGCG 1250
D  A  E  R  H  G  H  T  V  L  A  L  V  R  G  S  A
GCTAACTCCGACGGCGCGTCGAACGGTCTGTCGGCGCCGAACGGCCCCTC 1300
 A  N  S  D  G  A  S  N  G  L  S  A  P  N  G  P  S
CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350
  Q  E  R  V  I  H  Q  A  L  A  N  A  K  L  T  P
CCGATGTCGACGCGGTCGAGGCGCACGGCACCGGCACCCGCCTCGGCGAC 1400
A  D  V  D  A  V  E  A  H  G  T  G  T  R  L  G  D
CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGCGAC 1450
 P  I  E  A  Q  A  L  L  A  T  Y  G  Q  D  R  A  T
GCCCCTGCTGCTCGGCTCGCTGAAGTCGAACATCGGGCACGCCCAGGCCG 1500
  P  L  L  L  G  S  L  K  S  N  I  G  H  A  Q  A
CGTCAGGGGTCGCCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGGG 1550
A  S  G  V  A  G  I  I  K  M  V  Q  A  I  R  H  G
GAACTGCCGCCGACACTGCACGCGGACGAGCCGTCGCCGCACGTCGACTG 1600
 E  L  P  P  T  L  H  A  D  E  P  S  P  H  V  D  W
GACGGCCGGTGCCGTCGAGCTCCTGACGTCGGCCCGGCCGTGGCCGGGGA 1650
  T  A  G  A  V  E  L  L  T  S  A  R  P  W  P  G
CCGGTCGCCCGCGCCGCGCTGCCGTCTCGTCGTTCGGCGTGAGCGGCACG 1700
T  G  R  P  R  R  A  A  V  S  S  F  G  V  S  G  T
AACGCCCACATCATCCTTGAGGCAGGACCGGTCAAAACGGGACCGGTCGA 1750
 N  A  H  I  I  L  E  A  G  P  V  K  T  G  P  V  E
GGCAGGAGCGATCGAGGCAGGACCGGTCGAAGTAGGACCGGTCGAGGCTG 1800
  A  G  A  I  E  A  G  P  V  E  V  G  P  V  E  A
GACCGCTCCCCGCGGCGCCGCCGTCAGCACCGGGCGAAGACCTTCCGCTG 1850
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G   P   L   P   A   A   P   P   S   A   P   G   E   D   L   P   L
CTCGTGTCGGCGCGTTCCCCGGAGGCACTCGACGAGCAGATCGGGCGCCT 1900
 L   V   S   A   R   S   P   E   A   L   D   E   Q   I   G   R   L
GCGCGCCTATCTCGACACCGGCCCGGGCGTCGACCGGGCGGCCGTGGCGC 1950
  R   A   Y   L   D   T   G   P   G   V   D   R   A   A   V   A
AGACACTGGCCCGGCGTACGCACTTCACCCACCGGGCCGTACTGCTCGGG 2000
Q   T   L   A   R   R   T   H   F   T   H   R   A   V   L   L   G
GACACCGTCATCGGCGCTCCCCCCGCGGACCAGGCCGACGAACTCGTCTT 2050
 D   T   V   I   G   A   P   P   A   D   Q   A   D   E   L   V   F
CGTCTACTCCGGTCAGGGCACCCAGCATCCCGCGATGGGCGAGCAGCTAG 2100
  V   Y   S   G   Q   G   T   Q   H   P   A   M   G   E   Q   L
CCGCCGCGTTCCCCGTCTTCGCGCGGATCCATCAGCAGGTGTGGGACCTG 2150
A   A   A   F   P   V   F   A   R   I   H   Q   Q   V   W   D   L
CTCGATGTGCCCGATCTGGAGGTGAACGAGACCGGTTACGCCCAGCCGGC 2200
 L   D   V   P   D   L   E   V   N   E   T   G   Y   A   Q   P   A
CCTGTTCGCAATGCAGGTGGCTCTGTTCGGGCTGCTGGAATCGTGGGGTG 2250
  L   F   A   M   Q   V   A   L   F   G   L   L   E   S   W   G
TACGACCGGACGCGGTGATCGGCCATTCGGTGGGTGAGCTTGCGGCTGCG 2300
V   R   P   D   A   V   I   G   H   S   V   G   E   L   A   A   A
TATGTGTCCGGGGTGTGGTCGTTGGAGGATGCCTGCACTTTGGTGTCGGC 2350
 Y   V   S   G   V   W   S   L   E   D   A   C   T   L   V   S   A
GCGGGCTCGTCTGATGCAGGCTCTGCCCGCGGGTGGGGTGATGGTCGCTG 2400
  R   A   R   L   M   Q   A   L   P   A   G   G   V   M   V   A
TCCCGGTCTCGGAGGATGAGGCCCGGGCCGTGCTGGGTGAGGGTGTGGAG 2450
V   P   V   S   E   D   E   A   R   A   V   L   G   E   G   V   E
ATCGCCGCGGTCAACGGCCCGTCGTCGGTGGTTCTCTCCGGTGATGAGGC 2500
 I   A   A   V   N   G   P   S   S   V   V   L   S   G   D   E   A
CGCCGTGCTGCAGGCCGCGGAGGGGCTGGGGAAGTGGACGCGGCTGGCGA 2550
  A   V   L   Q   A   A   E   G   L   G   K   W   T   R   L   A
CCAGCCACGCGTTCCATTCCGCCCGTATGGAACCCATGCTGGAGGAGTTC 2600
T   S   H   A   F   H   S   A   R   M   E   P   M   L   E   E   F
CGGGCGGTCGCCGAAGGCCTGACCTACCGGACGCCGCAGGTCTCCATGGC 2650
 R   A   V   A   E   G   L   T   Y   R   T   P   Q   V   S   M   A
CGTTGGTGATCAGGTGACCACCGCTGAGTACTGGGTGCGGCAGGTCCGGG 2700
  V   G   D   Q   V   T   T   A   E   Y   W   V   R   Q   V   R
ACACGGTCCGGTTCGGCGAGCAGGTGGCCTCGTACGAGGACGCCGTGTTC 2750
D   T   V   R   F   G   E   Q   V   A   S   Y   E   D   A   V   F
GTCGAGCTGGGTGCCGACCGGTCACTGGCCCGCCTGGTCGACGGTGTCGC 2800
 V   E   L   G   A   D   R   S   L   A   R   L   V   D   G   V   A
GATGCTGCACGGCGACCACGAAATCCAGGCCGCGATCGGCGCCCTGGCCC 2850
  M   L   H   G   D   H   E   I   Q   A   A   I   G   A   L   A
ACCTGTATGTCAACGGCGTCACGGTCGACTGGCCCGCGCTCCTGGGCGAT 2900
H   L   Y   V   N   G   V   T   V   D   W   P   A   L   L   G   D
GCTCCGGCAACACGGGTGCTGGACCTTCCGACATACGCCTTCCAGCACCA 2950
 A   P   A   T   R   V   L   D   L   P   T   Y   A   F   Q   H   Q
GCGCTACTGGCTCGAGTCGGCTCCCCCGGCCACGGCCGACTCGGGCCACC 3000
  R   Y   W   L   E   S   A   P   P   A   T   A   D   S   G   H
CCGTCCTCGGCACCGGAGTCGCCGTCGCCGGGTCGCCGGGCCGGGTGTTC 3050
P   V   L   G   T   G   V   A   V   A   G   S   P   G   R   V   F
ACGGGTCCCGTGCCCGCCGGTGCGGACCGCGCGGTGTTCATCGCCGAACT 3100
 T   G   P   V   P   A   G   A   D   R   A   V   F   I   A   E   L
GGCGCTCGCCGCCGCCGACGCCACCGACTGCGCCACGGTCGAACAGCTCG 3150
  A   L   A   A   A   D   A   T   D   C   A   T   V   E   Q   L
ACGTCACCTCCGTGCCCGGCGGATCCGCCCGCGGCAGGGCCACCGCGCAG 3200
```

D V T S V P G G S A R G R A T A Q
ACCTGGGTCGATGAACCCGCCGCCGACGGGCGGCGCCGCTTCACCGTCCA 3250
T W V D E P A A D G R R R F T V H
CACCCGCGTCGGCGACGCCCCGTGGACGCTGCACGCCGAGGGGGTTCTCC 3300
T R V G D A P W T L H A E G V L
GCCCCGGCCGCGTGCCCCAGCCCGAAGCCGTCGACACCGCCTGGCCCCCG 3350
R P G R V P Q P E A V D T A W P P
CCGGGCGCGGTGCCCGCGGACGGGCTGCCCGGGGCGTGGCGACGCGCGGA 3400
P G A V P A D G L P G A W R R A D
CCAGGTCTTCGTCGAAGCCGAAGTCGACAGCCCTGACGGCTTCGTGGCAC 3450
Q V F V E A E V D S P D G F V A
ACCCCGACCTGCTCGACGCGGTCTTCTCCGCGGTCGGCGACGGGAGCCGC 3500
H P D L L D A V F S A V G D G S R
CAGCCGACCGGATGGCGCGACCTCGCGGTGCACGCGTCGGACGCCACCGT 3550
Q P T G W R D L A V H A S D A T V
GCTGCGCGCCTGCCTCACCCGCCGCGACAGTGGTGTCGTGGAGCTCGCCG 3600
L R A C L T R R D S G V V E L A
CCTTCGACGGTGCCGGAATGCCGGTGCTCACCGCGGAGTCGGTGACGCTG 3650
A F D G A G M P V L T A E S V T L
GGCGAGGTCGCGTCGGCAGGCGGATCCGACGAGTCGGACGGTCTGCTTCG 3700
G E V A S A G G S D E S D G L L R
GCTTGAGTGGTTGCCGGTGGCGGAGGCCCACTACGACGGTGCCGACGAGC 3750
L E W L P V A E A H Y D G A D E
TGCCCGAGGGCTACACCCTCATCACCGCCACACACCCCGACGACCCCGAC 3800
L P E G Y T L I T A T H P D D P D
GACCCCACCAACCCCCACAACACACCCACACGCACCCACACACAAACCAC 3850
D P T N P H N T P T R T H T Q T T
ACGCGTCCTCACCGCCCTCCAACACCACCTCATCACCACCAACCACACCC 3900
R V L T A L Q H H L I T T N H T
TCATCGTCCACACCACCACCGACCCCCCAGGCGCCGCCGTCACCGGCCTC 3950
L I V H T T T D P P G A A V T G L
ACCCGCACCGCACAAAACGAACACCCCGGCCGCATCCACCTCATCGAAAC 4000
T R T A Q N E H P G R I H L I E T
CCACCACCCCCACACCCCACTCCCCCTCACCCAACTCACCACCCTCCACC 4050
H H P H T P L P L T Q L T T L H
AACCCCACCTACGCCTCACCAACAACACCCTCCACACCCCCCACCTCACC 4100
Q P H L R L T N N T L H T P H L T
CCCATCACCACCCACCACAACACCACCACAACCACCCCCAACACCCCACC 4150
P I T T H H N T T T T T P N T P P
CCTCAACCCCAACCACGCCATCCTCATCACCGGCGGCTCCGGCACCCTCG 4200
L N P N H A I L I T G G S G T L
CCGGCATCCTCGCCCGCCACCTCAACCACCCCCACACCTACCTCCTCTCC 4250
A G I L A R H L N H P H T Y L L S
CGCACACCACCACCCCCCACCACACCCGGCACCCACATCCCCTGCGACCT 4300
R T P P P P T T P G T H I P C D L
CACCGACCCCACCCAAATCACCCAAGCCCTCACCCACATACCACAACCCC 4350
T D P T Q I T Q A L T H I P Q P
TCACCGGCATCTTCCACACCGCCGCCACCCTCGACGACGCCACCCTCACC 4400
L T G I F H T A A T L D D A T L T
AACCTCACCCCCCAACACCTCACCACCACCCTCCAACCCAAAGCCGACGC 4450
N L T P Q H L T T T L Q P K A D A
CGCCTGGCACCTCCACCACCACACCCAAAACCAACCCCTCACCCACTTCG 4500
A W H L H H H T Q N Q P L T H F
TCCTCTACTCCAGCGCCGCCGCCACCCTCGGCAGCCCCGGCCAAGCCAAC 4550

```
V  L  Y  S  S  A  A  A  T  L  G  S  P  G  Q  A  N
TACGCCGCCGCCAACGCCTTCCTCGACGCCCTCGCCACCCACCGCCACAC 4600
 Y  A  A  A  N  A  F  L  D  A  L  A  T  H  R  H  T
CCAAGGACAACCCGCCACCACCATCGCCTGGGGCATGTGGCACACCACCA 4650
  Q  G  Q  P  A  T  T  I  A  W  G  M  W  H  T  T
CCACACTCACCAGCCAACTCACCGACAGCGACCGCGACCGCATCCGCCGC 4700
T  T  L  T  S  Q  L  T  D  S  D  R  D  R  I  R  R
GGCGGCTTCCTGCCGATCTCGGACGACGAGGGCATGC
 G  G  F  L  P  I  S  D  D  E  G  M
```

The *Nhe*I-*Xho*I hybrid FK-506 PKS module 8 containing the AT domain of module 13 of rapamycin is shown below.

```
GCATGCGGCTGTACGAGGCGGCACGGCGCACCGGAAGTCCCGTGGTGGTG 50
   M  R  L  Y  E  A  A  R  R  T  G  S  P  V  V  V
GCGGCCGCGCTCGACGACGCGCCGGACGTGCCGCTGCTGCGCGGGCTGCG 100
 A  A  A  L  D  D  A  P  D  V  P  L  L  R  G  L  R
GCGTACGACCGTCCGGCGTGCCGCCGTCCGGGAACGCTCTCTCGCCGACC 150
  R  T  T  V  R  R  A  A  V  R  E  R  S  L  A  D
GCTCGCCGTGCTGCCCGACGACGAGCGCGCCGACGCCTCCCTCGCGTTCG 200
R  S  P  C  C  P  T  T  S  A  P  T  P  P  S  R  S
TCCTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCGCCGAAGACAT 250
 S  W  N  S  T  A  T  V  L  G  H  L  G  A  E  D  I
CCCGGCGACGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300
  P  A  T  T  T  F  K  E  L  G  I  D  S  L  T  A
TCCAGCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350
V  Q  L  R  N  A  L  T  T  A  T  G  V  R  L  N  A
ACAGCGGTCTTCGACTTTCCGACGCCGCGCGCGCTCGCCGCGAGACTCGG 400
 T  A  V  F  D  F  P  T  P  R  A  L  A  A  R  L  G
CGACGAGCTGGCCGGTACCCGCGCGCCCGTCGCGGCCCGGACCGCGGCCA 450
  D  E  L  A  G  T  R  A  P  V  A  A  R  T  A  A
CCGCGGCCGCGCACGACGAACCGCTGGCGATCGTGGGCATGGCCTGCCGT 500
T  A  A  A  H  D  E  P  L  A  I  V  G  M  A  C  R
CTGCCGGGCGGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550
 L  P  G  G  V  A  S  P  Q  E  L  W  R  L  V  A  S
CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG 600
  G  T  D  A  I  T  E  F  P  A  D  R  G  W  D  V
ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650
D  A  L  Y  D  P  D  P  D  A  I  G  K  T  F  V  R
CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGGCGTTCTTCGG 700
 H  G  G  F  L  D  G  A  T  G  F  D  A  A  F  F  G
GATCAGCCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750
  I  S  P  R  E  A  L  A  M  D  P  Q  Q  R  V  L
TGGAGACGTCCTGGGAGGCGTTCGAAAGCGCGGGCATCACCCCGGACGCG 800
L  E  T  S  W  E  A  F  E  S  A  G  I  T  P  D  A
GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850
 A  R  G  S  D  T  G  V  F  I  G  A  F  S  Y  G  Y
CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900
  G  T  G  A  D  T  N  G  F  G  A  T  G  S  Q  T
GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
S  V  L  S  G  R  L  S  Y  F  Y  G  L  E  G  P  S
GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000
 V  T  V  D  T  A  C  S  S  S  L  V  A  L  H  Q  A
```

```
AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050
  G  Q  S  L  R  S  G  E  C  S  L  A  L  V  G  G
TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100
V  T  V  M  A  S  P  G  G  F  V  E  F  S  R  Q  R
GGGCTCGCGCCGGACGGGCGGGCGAAGGCGTTCGGCGCGGGCGCGGACGG 1150
 G  L  A  P  D  G  R  A  K  A  F  G  A  G  A  D  G
TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG 1200
  T  S  F  A  E  G  A  G  A  L  V  V  E  R  L  S
ACGCGGAGCGCCACGGCCACACCGTCCTCGCCCTCGTACGCGGCTCCGCG 1250
D  A  E  R  H  G  H  T  V  L  A  L  V  R  G  S  A
GCTAACTCCGACGGCGCGTCGAACGGTCTGTCGGCGCCGAACGGCCCCTC 1300
 A  N  S  D  G  A  S  N  G  L  S  A  P  N  G  P  S
CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350
  Q  E  R  V  I  H  Q  A  L  A  N  A  K  L  T  P
CCGATGTCGACGCGGTCGAGGCGCACGGCACCGGCACCCGCCTCGGCGAC 1400
A  D  V  D  A  V  E  A  H  G  T  G  T  R  L  G  D
CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGCGAC 1450
 P  I  E  A  Q  A  L  L  A  T  Y  G  Q  D  R  A  T
GCCCCTGCTGCTCGGCTCGCTGAAGTCGAACATCGGGCACGCCCAGGCCG 1500
  P  L  L  L  G  S  L  K  S  N  I  G  H  A  Q  A
CGTCAGGGGTCGCCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGGG 1550
A  S  G  V  A  G  I  I  K  M  V  Q  A  I  R  H  G
GAACTGCCGCCGACACTGCACGCGGACGAGCCGTCGCCGCACGTCGACTG 1600
 E  L  P  P  T  L  H  A  D  E  P  S  P  H  V  D  W
GACGGCCGGTGCCGTCGAGCTCCTGACGTCGGCCCGGCCGTGGCCGGGGA 1650
  T  A  G  A  V  E  L  L  T  S  A  R  P  W  P  G
CCGGTCGCCCGCGCCGCGCTGCCGTCTCGTCGTTCGGCGTGAGCGGCACG 1700
T  G  R  P  R  R  A  A  V  S  S  F  G  V  S  G  T
AACGCCCACATCATCCTTGAGGCAGGACCGGTCAAAACGGGACCGGTCGA 1750
 N  A  H  I  I  L  E  A  G  P  V  K  T  G  P  V  E
GGCAGGAGCGATCGAGGCAGGACCGGTCGAAGTAGGACCGGTCGAGGCTG 1800
  A  G  A  I  E  A  G  P  V  E  V  G  P  V  E  A
GACCGCTCCCCGCGGCGCCGCCGTCAGCACCGGGCGAAGACCTTCCGCTG 1850
G  P  L  P  A  A  P  P  S  A  P  G  E  D  L  P  L
CTCGTGTCGGCGCGTTCCCCGGAGGCACTCGACGAGCAGATCGGGCGCCT 1900
 L  V  S  A  R  S  P  E  A  L  D  E  Q  I  G  R  L
GCGCGCCTATCTCGACACCGGCCCGGGCGTCGACCGGGCGGCCGTGGCGC 1950
  R  A  Y  L  D  T  G  P  G  V  D  R  A  A  V  A
AGACACTGGCCCGGCGTACGCACTTCACCCACCGGGCCGTACTGCTCGGG 2000
Q  T  L  A  R  R  T  H  F  T  H  R  A  V  L  L  G
GACACCGTCATCGGCGCTCCCCCCGCGGACCAGGCCGACGAACTCGTCTT 2050
 D  T  V  I  G  A  P  P  A  D  Q  A  D  E  L  V  F
CGTCTACTCCGGTCAGGGCACCCAGCATCCCGCGATGGGCGAGCAGCTAG 2100
  V  Y  S  G  Q  G  T  Q  H  P  A  M  G  E  Q  L
CCGATTCGTCGGTGGTGTTCGCCGAGCGGATGGCCGAGTGTGCGGCGGCG 2150
A  D  S  S  V  V  F  A  E  R  M  A  E  C  A  A  A
TTGCGCGAGTTCGTGGACTGGGATCTGTTCACGGTTCTGGATGATCCGGC 2200
 L  R  E  F  V  D  W  D  L  F  T  V  L  D  D  P  A
GGTGGTGGACCGGGTTGATGTGGTCCAGCCCGCTTCCTGGGCGATGATGG 2250
  V  V  D  R  V  D  V  V  Q  P  A  S  W  A  M  M
TTTCCCTGGCCGCGGTGTGGCAGGCGGCCGGTGTGCGGCCGGATGCGGTG 2300
V  S  L  A  A  V  W  Q  A  A  G  V  R  P  D  A  V
ATCGGCCATTCGCAGGGTGAGATCGCCGCAGCTTGTGTGGCGGGTGCGGT 2350
 I  G  H  S  Q  G  E  I  A  A  A  C  V  A  G  A  V
```

```
GTCACTACGCGATGCCGCCCGGATCGTGACCTTGCGCAGCCAGGCGATCG 2400
  S  L  R  D  A  A  R  I  V  T  L  R  S  Q  A  I
CCCGGGGCCTGGCGGGCCGGGGCGCGATGGCATCCGTCGCCCTGCCCGCG 2450
A  R  G  L  A  G  R  G  A  M  A  S  V  A  L  P  A
CAGGATGTCGAGCTGGTCGACGGGGCCTGGATCGCCGCCCACAACGGGCC 2500
 Q  D  V  E  L  V  D  G  A  W  I  A  A  H  N  G  P
CGCCTCCACCGTGATCGCGGGCACCCCGGAAGCGGTCGACCATGTCCTCA 2550
  A  S  T  V  I  A  G  T  P  E  A  V  D  H  V  L
CCGCTCATGAGGCACAAGGGGTGCGGGTGCGGCGGATCACCGTCGACTAT 2600
T  A  H  E  A  Q  G  V  R  V  R  R  I  T  V  D  Y
GCCTCGCACACCCCGCACGTCGAGCTGATCCGCGACGAACTACTCGACAT 2650
 A  S  H  T  P  H  V  E  L  I  R  D  E  L  L  D  I
CACTAGCGACAGCAGCTCGCAGACCCCGCTCGTGCCGTGGCTGTCGACCG 2700
  T  S  D  S  S  S  Q  T  P  L  V  P  W  L  S  T
TGGACGGCACCTGGGTCGACAGCCCGCTGGACGGGGAGTACTGGTACCGG 2750
V  D  G  T  W  V  D  S  P  L  D  G  E  Y  W  Y  R
AACCTGCGTGAACCGGTCGGTTTCCACCCCGCCGTCAGCCAGTTGCAGGC 2800
 N  L  R  E  P  V  G  F  H  P  A  V  S  Q  L  Q  A
CCAGGGCGACACCGTGTTCGTCGAGGTCAGCGCCAGCCCGGTGTTGTTGC 2850
  Q  G  D  T  V  F  V  E  V  S  A  S  P  V  L  L
AGGCGATGGACGACGATGTCGTCACGGTTGCCACGCTGCGTCGTGACGAC 2900
Q  A  M  D  D  D  V  V  T  V  A  T  L  R  R  D  D
GGCGACGCCACCCGGATGCTCACCGCCCTGGCACAGGCCTATGTCCACGG 2950
 G  D  A  T  R  M  L  T  A  L  A  Q  A  Y  V  H  G
CGTCACCGTCGACTGGCCCGCCATCCTCGGCACCACCACAACCCGGGTAC 3000
  V  T  V  D  W  P  A  I  L  G  T  T  T  T  R  V
TGGACCTTCCGACCTACGCCTTCCAACACCAGCGGTACTGGCTCGAGTCG 3050
L  D  L  P  T  Y  A  F  Q  H  Q  R  Y  W  L  E  S
GCTCCCCCGGCCACGGCCGACTCGGGCCACCCCGTCCTCGGCACCGGAGT 3100
 A  P  P  A  T  A  D  S  G  H  P  V  L  G  T  G  V
CGCCGTCGCCGGGTCGCCGGGCCGGGTGTTCACGGGTCCCGTGCCCGCCG 3150
  A  V  A  G  S  P  G  R  V  F  T  G  P  V  P  A
GTGCGGACCGCGCGGTGTTCATCGCCGAACTGGCGCTCGCCGCCGCCGAC 3200
G  A  D  R  A  V  F  I  A  E  L  A  L  A  A  A  D
GCCACCGACTGCGCCACGGTCGAACAGCTCGACGTCACCTCCGTGCCCGG 3250
 A  T  D  C  A  T  V  E  Q  L  D  V  T  S  V  P  G
CGGATCCGCCCGCGGCAGGGCCACCGCGCAGACCTGGGTCGATGAACCCG 3300
  G  S  A  R  G  R  A  T  A  Q  T  W  V  D  E  P
CCGCCGACGGGCGGCGCCGCTTCACCGTCCACACCCGCGTCGGCGACGCC 3350
A  A  D  G  R  R  R  F  T  V  H  T  R  V  G  D  A
CCGTGGACGCTGCACGCCGAGGGGGTTCTCCGCCCCGGCCGCGTGCCCCA 3400
 P  W  T  L  H  A  E  G  V  L  R  P  G  R  V  P  Q
GCCCGAAGCCGTCGACACCGCCTGGCCCCCGCCGGGCGCGGTGCCCGCGG 3450
  P  E  A  V  D  T  A  W  P  P  P  G  A  V  P  A
ACGGGCTGCCCGGGGCGTGGCGACGCGCGGACCAGGTCTTCGTCGAAGCC 3500
D  G  L  P  G  A  W  R  R  A  D  Q  V  F  V  E  A
GAAGTCGACAGCCCTGACGGCTTCGTGGCACACCCCGACCTGCTCGACGC 3550
 E  V  D  S  P  D  G  F  V  A  H  P  D  L  L  D  A
GGTCTTCTCCGCGGTCGGCGACGGGAGCCGCCAGCCGACCGGATGGCGCG 3600
  V  F  S  A  V  G  D  G  S  R  Q  P  T  G  W  R
ACCTCGCGGTGCACGCGTCGGACGCCACCGTGCTGCGCGCCTGCCTCACC 3650
D  L  A  V  H  A  S  D  A  T  V  L  R  A  C  L  T
CGCCGCGACAGTGGTGTCGTGGAGCTCGCCGCCTTCGACGGTGCCGGAAT 3700
 R  R  D  S  G  V  V  E  L  A  A  F  D  G  A  G  M
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```
GCCGGTGCTCACCGCGGAGTCGGTGACGCTGGGCGAGGTCGCGTCGGCAG 3750
 P  V  L  T  A  E  S  V  T  L  G  E  V  A  S  A
GCGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTGCCGGTG 3800
G  G  S  D  E  S  D  G  L  L  R  L  E  W  L  P  V
GCGGAGGCCCACTACGACGGTGCCGACGAGCTGCCCGAGGGCTACACCCT 3850
 A  E  A  H  Y  D  G  A  D  E  L  P  E  G  Y  T  L
CATCACCGCCACACACCCCGACGACCCCGACGACCCCACCAACCCCCACA 3900
 I  T  A  T  H  P  D  D  P  D  D  P  T  N  P  H
ACACACCCACACGCACCCACACACAAACCACACGCGTCCTCACCGCCCTC 3950
N  T  P  T  R  T  H  T  Q  T  T  R  V  L  T  A  L
CAACACCACCTCATCACCACCAACCACACCCTCATCGTCCACACCACCAC 4000
 Q  H  H  L  I  T  T  N  H  T  L  I  V  H  T  T  T
CGACCCCCCAGGCGCCGCCGTCACCGGCCTCACCCGCACCGCACAAAACG 4050
 D  P  P  G  A  A  V  T  G  L  T  R  T  A  Q  N
AACACCCCGGCCGCATCCACCTCATCGAAACCCACCACCCCCACACCCCA 4100
E  H  P  G  R  I  H  L  I  E  T  H  H  P  H  T  P
CTCCCCCTCACCCAACTCACCACCCTCCACCAACCCCACCTACGCCTCAC 4150
 L  P  L  T  Q  L  T  T  L  H  Q  P  H  L  R  L  T
CAACAACACCCTCCACACCCCCCACCTCACCCCCATCACCACCCACCACA 4200
 N  N  T  L  H  T  P  H  L  T  P  I  T  T  H  H
ACACCACCACAACCACCCCCAACACCCCACCCCTCAACCCCAACCACGCC 4250
N  T  T  T  T  T  P  N  T  P  P  L  N  P  N  H  A
ATCCTCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCCGCCA 4300
 I  L  I  T  G  G  S  G  T  L  A  G  I  L  A  R  H
CCTCAACCACCCCCACACCTACCTCCTCTCCCGCACACCACCACCCCCCA 4350
 L  N  H  P  H  T  Y  L  L  S  R  T  P  P  P  P
CCACACCCGGCACCCACATCCCCTGCGACCTCACCGACCCCACCCAAATC 4400
T  T  P  G  T  H  I  P  C  D  L  T  D  P  T  Q  I
ACCCAAGCCCTCACCCACATACCACAACCCCTCACCGGCATCTTCCACAC 4450
 T  Q  A  L  T  H  I  P  Q  P  L  T  G  I  F  H  T
CGCCGCCACCCTCGACGACGCCACCCTCACCAACCTCACCCCCCAACACC 4500
 A  A  T  L  D  D  A  T  L  T  N  L  T  P  Q  H
TCACCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCTCCACCAC 4550
L  T  T  T  L  Q  P  K  A  D  A  A  W  H  L  H  H
CACACCCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCAGCGCCGC 4600
 H  T  Q  N  Q  P  L  T  H  F  V  L  Y  S  S  A  A
CGCCACCCTCGGCAGCCCCGGCCAAGCCAACTACGCCGCCGCCAACGCCT 4650
 A  T  L  G  S  P  G  Q  A  N  Y  A  A  A  N  A
TCCTCGACGCCCTCGCCACCCACCGCCACACCCAAGGACAACCCGCCACC 4700
F  L  D  A  L  A  T  H  R  H  T  Q  G  Q  P  A  T
ACCATCGCCTGGGGCATGTGGCACACCACCACCACACTCACCAGCCAACT 4750
 T  I  A  W  G  M  W  H  T  T  T  T  L  T  S  Q  L
CACCGACAGCGACCGCGACCGCATCCGCCGCGGCGGCTTCCTGCCGATCT 4800
 T  D  S  D  R  D  R  I  R  R  G  G  F  L  P  I
CGGACGACGAGGGCATGC
S  D  D  E  G  M
```

Example 3

Recombinant PKS Genes for 13-desmethoxy FK-506 and FK-520

The present invention provides a variety of recombinant PKS genes in addition to those described in Examples 1 and 2 for producing 13-desmethoxy FK-506 and FK-520

compounds. This Example provides the construction protocols for recombinant FK-520 and FK-506 (from *Streptomyces* sp. MA6858 (ATCC 55098), described in U.S. Patent Nos. 5,116,756, incorporated herein by reference) PKS genes in which the module 8 AT coding sequences have been replaced by either the *rap*AT3 (the AT domain from module 3 of the rapamycin PKS), *rap*AT12, *ery*AT1 (the AT domain from module 1 of the erythromycin (DEBS) PKS), or *ery*AT2 coding sequences. Each of these constructs provides a PKS that produces the 13-desmethoxy-13-methyl derivative, except for the rapAT12 replacement, which provides the 13-desmethoxy derivative, i.e., it has a hydrogen where the other derivatives have methyl.

Figure 7 shows the process used to generate the AT replacement constructs. First, a fragment of ~4.5 kb containing module 8 coding sequences from the FK-520 cluster of ATCC 14891 was cloned using the convenient restriction sites *Sac*I and *Sph*I (Step A in Figure 7). The choice of restriction sites used to clone a 4.0 - 4.5 kb fragment comprising module 8 coding sequences from other FK-520 or FK-506 clusters can be different depending on the DNA sequence, but the overall scheme is identical. The unique *Sac*I and *Sph*I restriction sites at the ends of the FK-520 module 8 fragment were then changed to unique *Bgl* II and *Nsi*I sites by ligation to synthetic linkers (described in the preceding Examples, see Step B of Figure 7). Fragments containing sequences 5' and 3' of the AT8 sequences were then amplified using primers, described above, that introduced either an *Avr*II site or an *Nhe*I site at two different KS/AT boundaries and an *Xho*I site at the AT/DH boundary (Step C of Figure 7). Heterologous AT domains from the rapamycin and erythromycin gene clusters were amplified using primers, as described above, that introduced the same sites as just described (Step D of Figure 7). The fragments were ligated to give hybrid modules with in-frame fusions at the KS/AT and AT/DH boundaries (Step E of Figure 7). Finally, these hybrid modules were ligated into the *Bam*HI and *Pst*I sites of the KC515 vector. The resulting recombinant phage were used to transform the FK-506 and FK-520 producer strains to yield the desired recombinant cells, as described in the preceding Examples.

The following table shows the location and sequences surrounding the engineered site of each of the heterologous AT domains employed. The FK-506 hybrid construct was used as a control for the FK-520 recombinant cells produced, and a similar FK-520 hybrid construct was used as a control for the FK-506 recombinant cells.

Heterologous AT	Enzyme	Location of Engineered Site
FK-506 AT8 (hydroxymalonyl)	*Avr*II	GGCCGTccgcgcCGTGCGGCGGTCTCGTCGTTC G R P R R A A V S S F
	*Nhe*I	ACCCAGCATCCCGCGATGGGTGAGCGgctcgcC T Q H P A M G E R L A
	*Xho*I	TACGCCTTCCAGCGGCGGCCCTACTGGatcgag Y A F Q R R P Y W I E
rapamycin AT3 (methylmalonyl)	*Avr*II	GACCGGccccgtCGGGCGGGCGTGTCGTCCTTC D R P R R A G V S S F
	*Nhe*I	TGGCAGTGGCTGGGGATGGGCAGTGCcctgcgG W Q W L G M G S A L R
	*Xho*I	TACGCCTTCCAACACCAGCGGTACTGGgtcgag Y A F Q H Q R Y W V E
rapamycin AT12 (malonyl)	*Avr*II	GGCCGAgcgcgcCGGGCAGGCGTGTCGTCCTTC G R A R R A G V S S F
	*Nhe*I	TCGCAGCGTGCTGGCATGGGTGAGGAactggcC S Q R A G M G E E L A
	*Xho*I	TACGCCTTCCAGCACCAGCGCTACTGGctcgag Y A F Q H Q R Y W L E
DEBS AT1 (methylmalonyl)	*Avr*II	GCGCGAccgcgcCGGGCGGGGGTCTCGTCGTTC A R P R R A G V S S F
	*Nhe*I	TGGCAGTGGGCGGGCATGGCCGTCGAcctgctC W Q W A G M A V D L L
	*Xho*I	TACCCGTTCCAGCGCGAGCGCGTCTGGctcgaa Y P F Q R E R V W L E
DEBS AT2 (methylmalonyl)	*Avr*II	GACGGGgtgcgcCGGGCAGGTGTGTCGGCGTTC D G V R R A G V S A F
	*Nhe*I	GCCCAGTGGGAAGGCATGGCGCGGGAgttgttG A Q W E G M A R E L L
	*Xho*I	TATCCTTTCCAGGGCAAGCGGTTCTGGctgctg Y P F Q G K R F W L L

The sequences shown below provide the location of the KS/AT boundaries chosen in the FK-520 module 8 coding sequences. Regions where *Avr*II and *Nhe*I sites were engineered are indicated by lower case and underlining.

```
CCGGCGCCGTCGAACTGCTGACGTCGGCCCGGCCGTGGCCCGAGACCGACCGGccacggC
A  G  A  V  E  L  L  T  S  A  R  P  W  P  E  T  D  R  P  R
GTGCCGCCGTCTCCTCGTTCGGGGTGAGCGGCACCAACGCCCACGTCATCCTGGAGGCCG
R  A  A  V  S  S  F  G  V  S  G  T  N  A  H  V  I  L  E  A
GACCGGTAACGGAGACGCCCGCGGCATCGCCTTCCGGTGACCTTCCCCTGCTGGTGTCGG
G  P  V  T  E  T  P  A  A  S  P  S  G  D  L  P  L  L  V  S
CACGCTCACCGGAAGCGCTCGACGAGCAGATCCGCCGACTGCGCGCCTACCTGGACACCA
A  R  S  P  E  A  L  D  E  Q  I  R  R  L  R  A  Y  L  D  T
CCCCGGACGTCGACCGGGTGGCCGTGGCACAGACGCTGGCCCGGCGCACACACTTCGCCC
T  P  D  V  D  R  V  A  V  A  Q  T  L  A  R  R  T  H  F  A
ACCGCGCCGTGCTGCTCGGTGACACCGTCATCACCACACCCCCCGCGGACCGGCCCGACG
H  R  A  V  L  L  G  D  T  V  I  T  T  P  P  A  D  R  P  D
AACTCGTCTTCGTCTACTCCGGCCAGGGCACCCAGCATCCCGCGATGGGCGAGCAgctcg
E  L  V  F  V  Y  S  G  Q  G  T  Q  H  P  A  M  G  E  Q  L
cCGCCGCCCATCCCGTGTTCGCCGACGCCTGGCATGAAGCGCTCCGCCGCCTTGACAACC
A  A  A  H  P  V  F  A  D  A  W  H  E  A  L  R  R  L  D  N
```

The sequences shown below provide the location of the AT/DH boundary chosen in the FK-520 module 8 coding sequences. The region where an *Xho*I site was engineered is indicated by lower case and underlining.

```
TCCTCGGGGCTGGGTCACGGCACGACGCGGATGTGCCCGCGTACGCGTTCCAACGGCGGC
I  L  G  A  G  S  R  H  D  A  D  V  P  A  Y  A  F  Q  R  R
ACTACTGGatcgagTCGGCACGCCCGGCCGCATCCGACGCGGGCCACCCCGTGCTGGGCT
H  Y  W  I  E  S  A  R  P  A  A  S  D  A  G  H  P  V  L  G
```

The sequences shown below provide the location of the KS/AT boundaries chosen in the FK-506 module 8 coding sequences. Regions where *Avr*II and *Nhe*I sites were engineered are indicated by lower case and underlining.

```
TCGGCCAGGCCGTGGCCGCGGACCGGCCGTccgcgcCGTGCGGCGGTCTCGTCGTTCGGG
 S  A  R  P  W  P  R  T  G  R  P  R  R  A  A  V  S  S  F  G
GTGAGCGGCACCAACGCCCACATCATCCTGGAGGCCGGACCCGACCAGGAGGAGCCGTCG
 V  S  G  T  N  A  H  I  I  L  E  A  G  P  D  Q  E  E  P  S
GCAGAACCGGCCGGTGACCTCCCGCTGCTCGTGTCGGCACGGTCCCCGGAGGCACTGGAC
 A  E  P  A  G  D  L  P  L  L  V  S  A  R  S  P  E  A  L  D
GAGCAGATCGGGCGCCTGCGCGACTATCTCGACGCCGCCCCCGGCGTGGACCTGGCGGCC
 E  Q  I  G  R  L  R  D  Y  L  D  A  A  P  G  V  D  L  A  A
GTGGCGCGGACACTGGCCACGCGTACGCACTTCTCCCACCGCGCCGTACTGCTCGGTGAC
 V  A  R  T  L  A  T  R  T  H  F  S  H  R  A  V  L  L  G  D
ACCGTCATCACCGCTCCCCCCGTGGAACAGCCGGGCGAGCTCGTCTTCGTCTACTCGGGA
 T  V  I  T  A  P  P  V  E  Q  P  G  E  L  V  F  V  Y  S  G
CAGGGCACCCAGCATCCCGCGATGGGTGAGCGgctcgcCGCAGCCTTCCCCGTGTTCGCC
 Q  G  T  Q  H  P  A  M  G  E  R  L  A  A  A  F  P  V  F  A
```

```
GACCCGGACGTACCCGCCTACGCCTTCCAGCGGCGGCCCTACTGGATCGAGTCCGCGCCG
 D  P  D  V  P  A  Y  A  F  Q  R  R  P  Y  W  I  E  S  A  P
```

The sequences shown below provide the location of the AT/DH boundary chosen in the FK-506 module 8 coding sequences. The region where an *Xho*I site was engineered is indicated by lower case and underlining.

```
GACCCGGACGTACCCGCCTACGCCTTCCAGCGGCGGCCCTACTGGatcgagTCCGCGCCG
 D  P  D  V  P  A  Y  A  F  Q  R  R  P  Y  W  I  E  S  A  P
```

Example 4

Replacement of Methoxyl with Hydrogen or Methyl at C-15 of FK-506 and FK-520

The methods and reagents of the present invention also provide novel FK-506 and FK-520 derivatives in which the methoxy group at C-15 is replaced by a hydrogen or methyl. These derivatives are produced in recombinant host cells of the invention that express recombinant PKS enzymes the produce the derivatives. These recombinant PKS enzymes are prepared in accordance with the methodology of Examples 1 and 2, with the exception that AT domain of module 7, instead of module 8, is replaced. Moreover, the present invention provides recombinant PKS enzymes in which the AT domains of both modules 7 and 8 have been changed. The table below summarizes the various compounds provided by the present invention.

Compound	C-13	C-15	Derivative Provided
FK-506	hydrogen	hydrogen	13, 15-didesmethoxy-FK-506
FK-506	hydrogen	methoxy	13-desmethoxy-FK-506
FK-506	hydrogen	methyl	13,15-didesmethoxy-15-methyl-FK-506
FK-506	methoxy	hydrogen	15-desmethoxy-FK-506
FK-506	methoxy	methoxy	Original Compound -- FK-506
FK-506	methoxy	methyl	15-desmethoxy-15-methyl-FK-506
FK-506	methyl	hydrogen	13,15-didesmethoxy-13-methyl-FK-506
FK-506	methyl	methoxy	13-desmethoxy-13-methyl-FK-506
FK-506	methyl	methyl	13,15-didesmethoxy-13,15-dimethyl-FK-506
FK-520	hydrogen	hydrogen	13, 15-didesmethoxy FK-520

FK-520	hydrogen	methoxy	13-desmethoxy FK-520
FK-520	hydrogen	methyl	13,15-didesmethoxy-15-methyl-FK-520
FK-520	methoxy	hydrogen	15-desmethoxy-FK-520
FK-520	methoxy	methoxy	Original Compound -- FK-520
FK-520	methoxy	methyl	15-desmethoxy-15-methyl-FK-520
FK-520	methyl	hydrogen	13,15-didesmethoxy-13-methyl-FK-520
FK-520	methyl	methoxy	13-desmethoxy-13-methyl-FK-520
FK-520	methyl	methyl	13,15-didesmethoxy-13,15-dimethyl-FK-520

Example 5

Replacement of Methoxyl with Ethyl at C-13 and/or C-15 of FK-506 and FK-520

The present invention also provides novel FK-506 and FK-520 derivative compounds in which the methoxy groups at either or both the C-13 and C-15 positions are instead ethyl groups. These compounds are produced by novel PKS enzymes of the invention in which the AT domains of modules 8 and/or 7 are converted to ethylmalonyl specific AT domains by modification of the PKS gene that encodes the module. Ethylmalonyl specific AT domain coding sequences can be obtained from, for example, the FK-520 PKS genes, the niddamycin PKS genes, and the tylosin PKS genes. The novel PKS genes of the invention include not only those in which either or both of the AT domains of modules 7 and 8 have been converted to ethylmalonyl specific AT domains but also those in which one of the modules is converted to an ethylmalonyl specific AT domain and the other is converted to a malonyl specific or a methylmalonyl specific AT domain.

Example 6

Neurotrophic Compounds

The compounds described in Examples 1 - 4, inclusive have immunosuppressant activity and can be employed as immunosuppressants in a manner and in formulations similar to those employed for FK-506. The compounds of the invention are generally effective for the prevention of organ rejection in patients receiving organ transplants and

in particular can be used for immunosuppression following orthotopic liver transplantation. These compounds also have pharmacokinetic properties and metabolism that are more advantageous for certain applications relative to those of FK-506 or FK-520. These compounds are also neurotrophic; however, for use as neurotrophins, it is desirable to modify the compounds to diminish or abolish their immunosuppressant activity. This can be readily accomplished by hydroxylating the compounds at the C-18 position using established chemical methodology or novel FK-520 PKS genes provided by the present invention.

Thus, in one aspect, the present invention provides a method for stimulating nerve growth that comprises administering a therapeutically effective dose of 18-hydroxy-FK-520. In another embodiment, the compound administered is a C-18,20-dihydroxy-FK-520 derivative. In another embodiment, the compound administered is a C-13-desmethoxy and/or C-15-desmethoxy 18-hydroxy-FK-520 derivative. In another embodiment, the compound administered is a C-13-desmethoxy and/or C-15-desmethoxy 18,20-dihydroxy-FK-520 derivative. In other embodiments, the compounds are the corresponding analogs of FK-506. The 18-hydroxy compounds of the invention can be prepared chemically, as described in U.S. Patent No. 5,189,042, incorporated herein by reference, or by fermentation of a recombinant host cell provided by the present invention that expresses a recombinant PKS in which the module 5 DH domain has been deleted or rendered non-functional.

The chemical methodology is as follows. A compound of the invention (~200 mg) is dissolved in 3 mL of dry methylene chloride and added to 45 μL of 2,6-lutidine, and the mixture stirred at room temperature. After 10 minutes, tert-butyldimethylsilyl trifluoromethanesulfonate (64 μL) is added by syringe. After 15 minutes, the reaction mixture is diluted with ethyl acetate, washed with saturated bicarbonate, washed with brine, and the organic phase dried over magnesium sulfate. Removal of solvent *in vacuo* and flash chromatography on silica gel (ethyl acetate: hexane (1:2) plus 1% methanol) gives the protected compound, which is dissolved in 95% ethanol (2.2 mL) and to which is added 53 μL of pyridine, followed by selenium dioxide (58 mg). The flask is fitted with a water condenser and heated to 70°C on a mantle. After 20 hours, the mixture is

cooled to room temperature, filtered through diatomaceous earth, and the filtrate poured into a saturated sodium bicarbonate solution. This is extracted with ethyl acetate, and the organic phase is washed with brine and dried over magnesium sulfate. The solution is concentrated and purified by flash chromatography on silica gel (ethyl acetate: hexane (1:2) plus 1% methanol) to give the protected 18-hydroxy compound. This compound is dissolved in acetonitrile and treated with aqueous HF to remove the protecting groups. After dilution with ethyl acetate, the mixture is washed with saturated bicarbonate and brine, dried over magnesium sulfate, filtered, and evaporated to yield the18-hydroxy compound. Thus, the present invention provides the C-18-hydroxyl derivatives of the compounds described in Examples 1 - 4.

Those of skill in the art will recognize that other suitable chemical procedures can be used to prepare the novel 18-hydroxy compounds of the invention. See, e.g., Kawai *et al.*, Jan. 1993, Structure-activity profiles of macrolactam immunosuppressant FK-506 analogues, *FEBS Letters 316*(2): 107-113, incorporated herein by reference. These methods can be used to prepare both the C18-[*S*]-OH and C18-[*R*]-OH enantiomers, with the *R* enantiomer showing a somewhat lower IC_{50}, which may be preferred in some applications. See Kawai *et al.*, *supra*. Another preferred protocol is described in Umbreit and Sharpless, 1977, JACS 99(16): 1526-28, although it may be preferable to use 30 equivalents each of SeO_2 and t-BuOOH rather than the 0.02 and 3-4 equivalents, respectively, described in that reference.

All scientific and patent publications referenced herein are hereby incorporated by reference. The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments, that the foregoing description and example is for purposes of illustration and not limitation of the following claims.